OBSERVATIONS ON MEGAKARYOCYTOPOIESIS AND THROMBOCYTOPOIESIS IN CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Aan Marijke en onze kinderen

CONTENTS

Idiopathic thrombocytopenic purpura. In this thesis the use of this term without the adjective "acute" refers to the chronic form of this disease.

Total megakaryocyte volume: the fractional volume of the bone marrow occupied by megakaryocytes.
This can refer to the total bone marrow space of the body or to a marrow sample. Without other indications the term total megakaryocyte volume concerns a bone marrow sample.

Serial section study: a study of all the consecutive histological sections (microtome slices of tissue).

Random section study: a study of some randomly selected histological sections.

(true) Diameter of a megakaryocyte: the maximum diameter of a megakaryocyte. If the diameter of a megakaryocyte is more than the thickness of the histological section then the (true) diameter of the megakaryocyte is equal to the diameter of the largest section of the megakaryocyte in a serial section study.

(Megakaryocyte) section diameter: diameter of the profile of a (cross-) section of a megakaryocyte in a histological section.

Cellularity: the number of cells per tissue volume unit.

Labelling index: the percentage of cells labelled with a radioactive marker.

GENERAL INTRODUCTION

It is rather generally accepted that thrombocytopenia in patients with chronic idiopathic thrombocytopenic purpura (ITP) is mainly caused by a shortened platelet survival. The influence of platelet production on the development of this kind of thrombocytopenia is not clear. Reports of platelet production, calculated from platelet survival in ITP patients are contradictory:increased, normal and decreased platelet productions are reported. This can points to the fact that quantitative or qualitative alterations in megakaryocytopoiesis is a pathogenetic factor in ITP,at least in some of these patients.

The aim of the present study was to improve our knowledge of ITP,especially concerning megakaryocytopoiesis and the effectiveness of thrombocytopoiesis. Therefore some quantitative and qualitative aspects of megakaryocytopoiesis and thrombocytopoiesis were examined with more recent methods of examination.

Quantitative analysis of megakaryocytopoiesis was performed using histometrical determinations of the total megakaryocyte volume in bone marrow biopsies and using measurements of relative deoxyribonucleic acid (DNA) content of megakaryocytic nuclei in bone marrow smears. Until now the morphometric method employed, has not been used for quantitation of megakaryocytes. Therefore the sources of error will be discussed in full. Measurements of relative megakaryocytic DNA content were carried out by micro-fluorometry. Polyploidy levels of megakaryocytes were calculated with the relative DNA content of other somatic (diploid) cells as a reference. The significance of ploidy levels for megakaryocytic size and for the degree of stimulation of thrombocytopoiesis will be discussed. The quantitative analysis of the total megakaryocyte volume of bone marrow tissue and the analysis of the ploidy of megakaryocytes were performed in order to gain some insight into the potential platelet generating capacity and into the degree of stimulation of megakaryocytopoiesis in patients with chronic ITP.

Qualitative insight into megakaryocytopoiesis was achieved by autoradiographic studies of megakaryocytes incubated with ^{3}H-thymidine and by studying the maturation stages of the megakaryocytes. Combined determinations of the labelling-

index of megakaryocytes and the distribution of megakaryocytes in the various maturation classes gave information about the rate of maturation in chronic ITP in comparison with other forms of thrombocytopenia. Furthermore, some insight into qualitative aspects of megakaryocytopoiesis was obtained by analysis of autoradiographs of megakaryocytes incubated with ^{3}H-uridine and ^{3}H-methionine, which gave information about ribonucleic acid and protein synthesis. These qualitative approaches to megakaryocyte- activity in patients with chronic ITP gave further information of the effectiveness of thrombocytopoiesis.

Another indirect method of studying the megakaryocyte function in ITP was provided by the exact determination of the volume of circulating platelets. Finally the effective platelet production was examined using a retrospective study in chronic ITP patients who were splenectomized. The rise in platelet counts in ITP patients immediately after splenectomy was compared with platelet counts in other splenectomized patients.

The results, obtained with the above mentioned methods, concerning quantitative and qualitative aspects of megakaryocytopoiesis in patients with chronic ITP suggest that ITP is a syndrome and not a clinical entity. An attempt has been made to distinguish different types of ITP in order to give improved indications regarding therapy and prognosis.

CHAPTER 1

THROMBOCYTOPOIESIS AND MEGAKARYOCYTOPOIESIS

1.1. THROMBOCYTOPOIESIS AND MEGAKARYOCYTOPOIESIS UNDER NORMAL CONDITIONS

1.1.1.Thrombocytopoiesis and thrombocytokinetics

Platelets develop from megakaryocytes. This was suggested as early as 1906 by Wright and was later demonstrated by electronic microscopic examinations (Kautz 1955;Yamada 1957) and by the cytochemical (Silber et al. 1960) and antigenic immunological relationship between platelets and megakaryocytes (Vaquez et al. 1960).
Blood platelet counts are rather constant in man (Brecher et al. 1953;Morley 1969a). Normal values vary between 150 and 450 x $10^3/\mu l$. These differences are probably the result of differences between the counting techniques used.

After release from the bone marrow, about 70% of the platelets can be found circulating in the blood and about 30% is enclosed within the spleen (Aster, 1966). With an increase in the size of the spleen there is an increase in the splenic platelet pool (Kutti et al. 1972). Platelets in the vascular pool and splenic pool are exchangeable (Aster 1966;Harker 1971a).

Reports of platelet life span under normal conditions studied with various techniques vary between 7 and 14 days (Bosch 1965,Harker et al. 1969;Najean et al. 1969, 1972;Kotilainen 1969, 1971;Kummer 1972;Paulus 1974). In all methods for estimation of platelet life span,platelets are labelled with radioactive markers while afterwards the disappearance rate of those markers from the blood is measured. Leeksma and Cohen (1956) were the first to employ this method, using ^{32}P -di-isopropylfluorophosphate for in vivo labelling of platelets. In later years ^{51}Cr -sodium chromate (Aas and Gardner 1958;Aster 1969), ^{14}C -serotonin (Heyssel 1968), ^{35}S sodium sulphate (Odell et al. 1967a;Roozendaal 1973) and ^{75}Se -methionine (Najean and Ardaillou 1969; Brodsky et al. 1972) have been em-

ployed. The way in which platelets disappear, by ageing or consumption, is not clear. In recent publications it is postulated that the ageing of platelets increases with the degree of their activity. This led to a mathematical analysis of platelet survival curves of labelled platelets based on the "multiple hits model"(Mustard et al. 1966;Murphy 1971;Paulus 1974). With this model exponential curves and linear curves as well as many intermediate forms can be explained by varying the number of hits needed for platelet death and the number of hits per time unit. Recent surveys of the methodology of platelet labelling, evaluation of recovery, survival and turnover are given by Kummer (1972) and Paulus (1971, 1974).

Until recently it was generally accepted that young platelets were large platelets and that platelet volume decreased with the ageing of the platelets (Detwiler et al. 1962;Mc. Donald et al. 1964;Karpatkin et al. 1969a,b;Garg et al. 1971, 1972;Kraytman, 1973). Recent studies by Paulus (1974) proved that platelet volume is not age dependent but is determined by the manner of platelet formation in megakaryocytes.

At present there are no reliable methods for direct determination of platelet production. However there are various indirect approaches·

1. Estimation of platelet turnover

Under normal conditions there is an equilibrium between platelet turnover and platelet production. Platelet turnover can be calculated from the blood platelet count, corrected for splenic pooling, divided by the platelet life span. However, measurement of platelet life span is not reliable. Therefore the results of different techniques for labelling of platelets and the calculation of platelet turnover are rather divergent;varying from 30,000 - 70,000 platelets per microlitre of blood per day (Harker et al. 1969;Kotilainen 1969; Kutti et al. 1971; Kummer 1972; Paulus 1974).

2. Estimation of platelet radioactivity after in vivo incorporation by megakaryocytes

After incorporation of ^{35}S -sodium sulphate or ^{75}Se-methionine in megakaryocytes, labelled platelets are delivered into the circulation. From the course of the radioactivity of circulating platelets, platelet life span and platelet production can be calculated (Najean et al. 1969; Brodsky et al. 1970).

3. Estimation of platelet volume

Under normal conditions the mean platelet volume is 7 μ^3 with a range of 5 to 12 μ^3 (Bull et al. 1965; Karpatkin 1969; Baadenhuysen 1971). If there is an increased stimulation of thrombocytopoiesis the mean platelet volume is increased.

Therefore the mean platelet volume is a rough indication of platelet production.

4. Estimation of total megakaryocyte volume

The total megakaryocyte volume or megakaryocyte mass is defined by the product of the number of megakaryocytes and their individual volumes. Harker et al. (1969) and Kutti et al. (1973) found a direct and positive relationship between platelet turnover rate and the total megakaryocyte volume. So, with some provisos, the total megakaryocyte volume reflects the platelet generating capacity of the marrow.

1.1.2. Megakaryocytopoiesis

Normally, the development of megakaryocytes from stem cells and their maturation occurs in the bone marrow. Megakaryocytes are also found in the circulating blood, more frequently in the central venous blood than in the venous blood of the pulmonary circulation. So pulmonary megakaryocytes (Breslow et al. 1968; Ikkala et al. 1972) probably do not arise in the lung (Kaufman et al. 1956b). Extramedullary megakaryocytes seem to be of inconsiderable importance in platelet production when there are no myeloproliferative diseases with myeloid metaplasia (Ebbe 1968c; Harker 1970b).

Knowledge regarding the development and maturation of megakaryocytes has been mainly obtained by autoradiographic examinations of marrow cells after injection of labelled thymidine under various experimental conditions. These results are supported by studies on the haemopoietic colonies which develop in spleens after injection of marrow cell suspensions in animals with an aplastic marrow (Ebbe et al. 1971; Nakeff 1972).

Current knowledge about megakaryocytopoiesis can be summarized as follows (fig. 1):

Pluripotential stem cells, which are not recognizable with standard morphological methods, develop into committed stem cells. These mononuclear committed stem cells, morphologically unrecognizable, can only develop up to megakaryocytes. Committed stem cells develop into polyploid precursor cells (Ebbe 1968c). Pluripotential stem cells, committed stem cells and precursor cells are proliferating cells. The subsequent stages of development of megakaryocytopoiesis are recognizable with standard staining techniques and can be divided into three stages of maturation (Bessis 1956; Feinendegen et al. 1962; Odell et al. 1968).

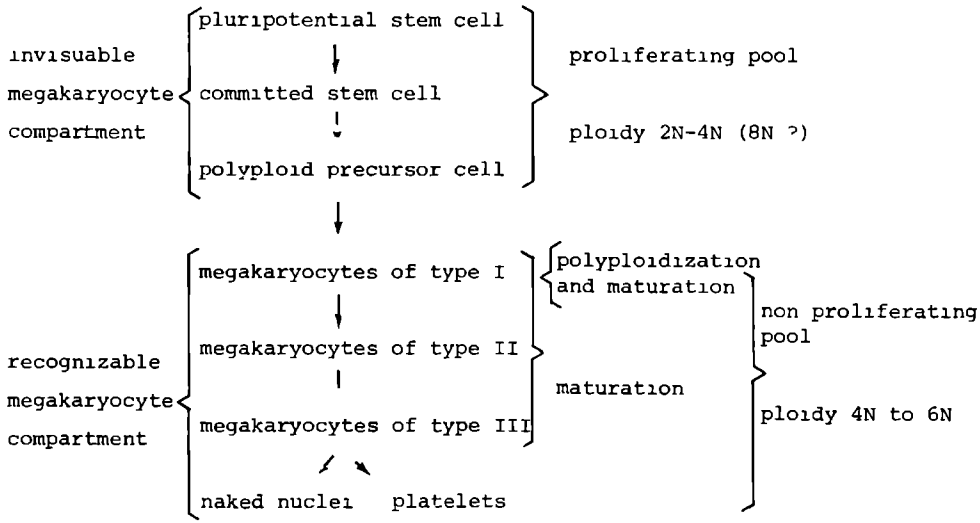

Fig. 1. Model of megakaryocytopoiesis.

In this study all recognizable cells of megakaryocytopoiesis will be denominated as megakaryocytes and differentiated in types I, II and III, using the classification of Feinendegen et al. (1962). In some other studies the term megakaryocyte is restricted to the last maturation stage of the recognizable cells of megakaryocytopoiesis while earlier maturation stages are indicated as megakaryoblasts and promegakaryocytes (Bessis 1956).

Megakaryocytes, as indicated by their name, are characterized by a large amount of nuclear material. This varies from between 2 and 32 times the normal amount of DNA of somatic cells which is constant (diploid; 2N). Sklarew et al. (1971) postulate that polyploidal cells, at least the first stages, develop from a fusion of lymphoid cells. However most authors accept that the polyploidy of megakaryocytes is the result of synchronous nuclear replications without cell division (Japa 1943; Garcia 1964; de Leval 1964, 1968a,b; Odell et al. 1965a). This process is called endoduplication or polyploidization. These nuclear replications occur in precursor cells and in the megakaryocytes with the most immature cytoplasm (Ebbe 1965; Odell et al. 1965a).

It is not known at which polyploidy level the most immature human megakaryocytes become morphologically recognizable. In animals this occurs at the 2N and 4N

levels (Odell et al. 1968) or at the most when the 8N level is reached (Paulus 1974). Until recently it was accepted that the maturation of the cytoplasm occurred only when ploidization was finished because thymidine incorporation was only seen in megakaryocytes with the most immature cytoplasm (Feinendegen et al. 1962; Ebbe et al. 1965; de Leval 1966; Odell et al. 1968, 1971). Recently it became clear from studies with electron microscopic and autoradiographic techniques combined with determinations of DNA that allready in DNA synthesizing and endomitotic megakaryocytes early signs of cytoplasmic specialization, such as the formation of granules and demarcation membranes, occurred (Paulus 1970, 1974; Mac Pherson 1971).

Nuclei of DNA synthesizing megakaryocytes are practically round with cytoplasmic invaginations. These are only recognizable by electronic microscopy. After completion of the ploidization the nucleus develops to an irregular mass; a segmented nucleus which finally, after platelet release becomes pycnotic (Paulus 1974).

It is generally accepted that maturation of megakaryocytic cytoplasm and platelet formation is possible in megakaryocytes at all ploidy levels of more than 4N (Odell and Jackson 1968a; Paulus 1974). Stadhouders (1974) has reported platelet formation in 4N megakaryocytes in Guinea-pigs. The amount of cytoplasm increases during maturation. Granules and demarcation membranes develop in the cytoplasm (Yamada 1957; Odell and Jackson 1968a; Stadhouders 1974). Previously, it was accepted that the α granules are derived from Golgi vesicles (Jones 1960). But the recent observations of Stadhouders (1974): a synchronic appearance of α granules and demarcation membranes, the preference localisation of α granules in apposition to the demarcation membrane and the morphological continuity between demarcation membrane and the membranes of α granules, suggest a development of α granules from demarcation membranes. Finally, the cytoplasm is fragmented by the demarcation membranes and groups of granules are relaesed. The residual nuclear material is phagocytosed and destroyed by the reticuloendothelial cells.

The maturation time of megakaryocytes from various animals proved to be 2 to 4 days. This was studied by means of "flash-labelling" of megakaryocytic nuclei (Ebbe et al. 1965; Odell et al. 1969a). In man there are no such exact determinations of maturation time available. Based on a) the results of labelling studies (Cronkite et al. 1961; Clarkson et al. 1971) b) the lag period between sti-

mulation of thrombocytopoiesis and increased platelet production (Krevans et al. 1955) and c) calculations of megakaryocytic turnover from the total megakaryocyte volume and platelet production (Harker et al. 1969), the maturation time of human megakaryocytes is thought to be 4 to 6 days

The number of human megakaryocytes is 6.1 x 10^6 per kg. body weight according to Harker et al. (1969) using an isotope dilution method. Determinations of megakaryocytic volume are only done on fixed material, so they do not have an absolute value. Quantitative aspects of megakaryocytopoiesis will be discussed in detail in the following chapter.

1.1.3. Regulation of thrombocytopoiesis and megakaryocytopoiesis

The rather constant blood platelet levels, the return to the original platelet level after an increase or decrease, e.g. in cases of blood loss and the simultaneous changes in megakaryocytopoiesis in those cases, indicates that there is a "feed back" mechanism (a regulatory mechanism) for platelet production. Many observations indicate that this regulatory mechanism is directed toward maintaining a constant number of platelets per kg body weight (Aster 1967; de Gabriele et al. 1967c) while some observations indicate that the aim of the regulatory mechanism is a constant level of circulating platelets (Harker et al. 1969, 1970c, 1971a). One or more humoral factors are decisive in this regulatory mechanism. A stimulating humoral thrombopoietic factor, thrombopoietin, has been demonstrated with certainty (Odell et al. 1961; Krizsa et al. 1969; Harker 1970e; Roosendaal 1973; Mc Donald 1973).

The time interval between an induced thrombocytopenia and an increased platelet production is about equal to the maturation time of megakaryocytes. This implies a short generation time of thrombopoietin in cases of an induced thrombocytopenia. Roosendaal (1973) demonstrated that thrombopoietin can be formed within 24 hours in rats. Many observations indicate that thrombopoietin influences megakaryocytopoiesis in different ways (Matter et al. 1960; Harker 1968b, 1969, 1970; Ebbe et al. 1968b, 1969, 1970a; Pennington et al. 1970; Rolovic et al. 1970; Nakeff 1972; Roosendaal 1973; Mac Pherson 1974).

The action of the humoral factor at the committed stem cell level results in an increase in megakaryocytes, their ploidy and their amount of cytoplasm. It is

not known if the development from committed stem cell to platelet generating megakaryocytes or platelet release is increased by the action of thrombopoietin. Some investigators believe in the existence of stimulating and suppressing humoral factors (Steinberg et al. 1965). While the existence of suppressing factors is very dubious (de Gabriele et al. 1967a; Harker 1968b), inactivation of thrombopoietin by platelets seems possible (de Gabriele 1967b).

Besides specific stimulation of thrombocytopoiesis there are also aspecific stimuli such as leucovorin and vincristine (Hayes et al. 1961; Rák 1972).

Under normal conditions platelet life span is not influenced by the spleen. This is demonstrated by a normal platelet survival after splenectomy (Leeksma et al. 1956). In man platelet count per kg body weight after splenectomy was normal according to Aster (1966, 1967). On the contrary Harker (1971a) found lower platelet counts per body weight in asplenic rats.

The transient thrombocytosis immediately following splenectomy is often explained by the decreased distribution volume for the circulating platelets and an aspecific effect of the surgical procedure. The distribution of platelets over the blood volume and splenic pool is changed in splenomegaly. The number of platelets in the splenic platelet pool then increases (Kutti et al. 1972). Moreover some investigators reported a slight decrease in platelet life span associated with splenomegaly (Aster 1966; Kotilainen 1969; Kutti et al. 1973), while Harker et al. (1969) reported a normal platelet life span. Shulman et al. (1965) observed a fall in platelet count within a few hours following administration of a spleen preparation and suggested that platelets were being removed more rapidly by the reticuloendothelial system.

Recent surveys of the regulation of megakaryocytopoiesis and thrombocytopoiesis are given by Ebbe (1970) and Harker (1970e).

In summary it can be concluded that platelet production seems to be regulated by humoral factors. The existence of a stimulating humoral factor, called thrombopoietin, is proven. In man it is uncertain if thrombopoietin generation depends on the circulating platelet concentration alone or on the platelet number per kg body weight which is inclusive of splenic platelets. The points of attack of thrombopoietin in megakaryocytopoiesis are known to some extent. The spleen may have an inhibitory effect on thrombocytopoiesis besides the well known platelet

pool function.

1.2. THROMBOCYTOPOIESIS AND MEGAKARYOCYTOPOIESIS IN CONDITIONS WITH ABNORMAL PLATELET COUNTS

1.2.1. Classification of abnormal platelet counts

Abnormal circulating platelet counts can be the effect of abnormal production, distribution or destruction. Sometimes there are also simultaneous qualitative platelet disorders, such as functional disorders (thrombopathy) which are outside the field of this study.

Thrombocytopenia

A decreased platelet count, a thrombocytopenia, can be caused by a decreased platelet production, a decreased platelet life span, or an increased pooling of platelets in the spleen. Sometimes more than one mechanism is simultaneously active.

A decreased platelet production can be caused by decreased megakaryocytic concentration. This rarely occurs in itself but mainly as part of an insufficiency of all haemato poietic cell lines (a bone marrow aplasia).

Sometimes the total megakaryocyte volume is relatively large in comparison to the platelet production. These conditions are defined as ineffective thrombocytopoiesis. In fact one can only prove the existence of an ineffective thrombocytopoiesis if one can prove that the maturation time of the megakaryocytes is not longer in these cases, or more generally if it is possible to compare the total megakaryocyte volume turnover rate and the turnover rate of the total circulating platelet mass with the product of the number of platelets and their individual volumes. This has never been reported. In cases of an ineffective thrombocytopoiesis the defect may be related to platelet formation, platelet release or intramedullary platelet destruction. These abnormalities may be congenital, as in some cases of familial thrombocytopenia, or acquired, as in patients with B_{12} or folate deficiency and Di Guglielmo's syndrome (Harker and Finch 1969).

An abnormal platelet distribution occurs in all cases of splenomegaly due to an increased pooling of platelets in the spleen. This may lead to a decreased

platelet count in the general circulation (Aster 1966). Sometimes 90% of all platelets are pooled in the spleen.

An increased platelet turnover can be the result of inferior platelet quality. More frequently an increased turnover is observed in cases with an increased platelet destruction caused by immunological mechanismsor by an increased platelet consumption by coagulation processes or direct platelet damage. Specific platelet destruction by immunological processes has been examined in detail. A well known form is the so called idiopathic thrombocytopenic purpura (ITP) which will be discussed separately in section 1.2.2. Moreover, immunological thrombocytopenia may be drug induced. The drug may acts as hapteen or by causing an immunological reaction by changing the platelet membrane. Auto-immune thrombocytopenias are kwown as e.g. in lupus erythematosus, mononucleosis and haemolytic anaemias. An increased platelet consumption, sometimes leading to a thrombocytopenia, is seen in cases of disseminated intravascular coagulation (DIC), in cases with abnormal vascular surfaces, e.g. caused by viral infections or atherosclerosis, in cases of acute promyelocytic leukaemia, hypersplenism or through phagocytotic activity of platelets in viral infections.

Good surveys of conditions with increased platelet consumption are given by Harker (1971c) and Paulus (1971, 1974).

Thrombocytosis

Thrombocytosis can be the result of a reactive or autonomous process. Reactive thrombocytosis is seen in patients with haemorrhagia, infectious diseases, malignant diseases (Hodgkin), chronic bowel diseases, rheumatoid arthritis, iron deficiency or temporarily after injections of vincristin (Selroos 1973). The platelets in cases of reactive or secondary thrombocytosis are functionally normal. Therefore those cases of thrombocytosis lead to an increased frequency of thrombosis .

Autonomous (essential or primary) thrombocytosis rarely occurs in itself. Generally these thrombocytosis is a stage before, or a part of polycythaemia vera, chronic myeloid leukaemia or osteomyelofibrosis. In spite of the increased platelet count a haemorrhagic diathesis may occur because the platelets are inferior.

1.2.2. Idiopathic thrombocytopenic purpura (ITP)

Definition

In this study ITP is defined by means of the following criteria:

1. a decreased platelet count in the blood
2. the absence of a primary disease which can lead to thrombocytopenia
3. the absence of an enlarged spleen
4. the absence of preceding drug use which could lead to thrombocytopenia

Pathological conditions with the above mentioned criteria are often characterized by the following laboratory findings which are however not obligatory for the diagnosis ITP:

1. a shortened platelet life span
2. an increase in the number of megakaryocytes
3. the existence of antiplatelet antibodies which cannot be explained by exogenous causes or primary diseases

If this clinical syndrome exists for more than 3 months we define it as chronic ITP. Acute ITP is characterized by data suggesting a different pathogenesis than in chronic ITP (Dameshek 1960; Baldini 1972). In acute ITP previous infectious diseases are often important in the pathogenesis (Lusher and Zuelzer 1966).

Some authors restrict the term ITP to conditions with a shortened platelet life span and/or the presence of antiplatelet antibodies. Others enlarge the definition of ITP by reckoning all immunological thrombocytopenias among ITP as long as the specificity of the various immunological processes is not demonstrated (Karpatkin 1971; Baldini 1972; Stijnen 1973).

Pathogenesis

It is generally well accepted that shortened platelet survival due to an increased destruction by the reticuloendothelial system, especially by the spleen, is an important factor in the pathogenesis of ITP while the role of platelet production is controversial (Karpatkin et al. 1971; Najean et al. 1973).

Platelet life span

Najean et al. (1973) in the largest described series of 135 ITP patients always

encountered a decreased platelet life span. Others occasionally observed a normal platelet life span (Cohen et al. 1961; Aster et al. 1964; Donaldson et al. 1971). These latter observations suggest decreased platelet production as a contributory cause in ITP.

Various arguments indicate an immunological, especially humoral cause of the decreased platelet life span:

1. Plasma of ITP patients is mostly capable of immediately inducing a transient thrombocytopenia in normal recipients (Harrington et al.1951)
2. In neonates of mothers with ITP a transient thrombocytopenia is often found (Epstein et al. 1950; Lusher and Zuelzer 1966)
3. Platelets tranfused into ITP patients disappear quickly from the circulation (Najean et al. 1973)
4. Antiplatelet antibodies, in the IgG globulin fraction of the blood, can be demonstrated with various kinds of techniques in about 70% of the ITP patients (Karpatkin et al. 1969, 1972a,b)
5. Treatment with immunosuppressive agents and corticosteroids is often successful in ITP patients (Harrington et al. 1951; Shulman et al. 1964; Karpatkin 1971a).

Recently, cellular immunity to autologous plateiets has also been suggested (Clancy 1972; Wybran et al. 1972).

ITP is often assumed to be an autoimmune disease since there are similarities with other autoimmune diseases and the cause of the development of the antiplatelet antibodies is idiopathic. However recently the autoimmune character was questioned by Nieweg et al. (1969) and Stijnen (1973) because in many cases they could not distinguish, by in vitro methods, between the antibodies in ITP and the antibodies in drug induced or post infectious thrombocytopenia. They suggested that multiple exogenous factors in those diseases can cause non-specific platelet damage ("spoiled membrane") which activates a common pathway of immunological platelet destruction. A change in the platelet membrane should be necessary for the activity of these antibodies. However with this "spoiled membrane allergy" hypothesis (Stijnen 1973) the transient thrombocytopenia in normal recipients following transfusion of ITP plasma (Harrington et al. 1951) and the frequency of ITP in young woman, as in other autoimmune diseases, is difficult to explain.

Site of platelet destruction

Studies with labelled platelets showed that in about 70% of the ITP patients the major site of platelet destruction is the spleen. In a minority of patients the liver or other sites in the reticuloendothelial system are more important sites of platelet destruction; possibly even intravascular destruction occurs (Najean et al. 1967; Fontein 1971; Aster 1971, 1972). Phagocytosis of platelets by splenic cells has been demonstrated by electronic microscopy (Mac Millan et al. 1974).

The role of the spleen

The spleen plays an important role in pathogenesis of ITP as it is often the major site of platelet destruction (Najean et al. 1971). Platelet survival time may return to normal after spelenctomy.

The antiplatelet antibody production in the spleen of ITP patients seems to be less important although this acitivity of the spleen is suggested by the much increased IgG synthesis activity of lymphoid cells from suspensions of spleen tissue (Mac Millan et al 1974). Moreover a significant decline in the serum anti-platelet antibody titre following splenectomy is seen in many patients with ITP (Karpatkin 1972a).

The platelet pooling in the spleen of patients with ITP is normal.

It is an old question if in ITP the spleen has an inhibitory effect on platelet production. This possibility has not been ruled out with certainty.

Platelet production

Studies concerning calculations of platelet productions from survival rates of labelled platelets in ITP are conflicting. Most observations indicate an increased platelet production, however in some studies a normal or even a decreased platelet production was calculated in some patients (Cohen et al. 1961; Ebbe et al. 1963; Aster et al. 1964a, 1966b; Davey 1966; Baldini 1966; Najean et al. 1963, 1969; Harker 1970c; Brodsky et al. 1972; Paulus 1974). The great differences in reported platelet production is illustrated by comparing the studies of Najean et al. (1963) concerning 85 untreated patients and Harker (1970c) who studied 16 patients. They calculated respectively that the mean platelet production was 1.3 and 4 times the normal value, with a range of 0.5 to 8. These

conflicting results of platelet production calculated with platelet survival studies are probably the result of the differences between these methods and the inaccuracy of all methods (Brodsky et al. 1972).

Another approach to the problem of the eventually decreased platelet production in ITP is a qualitative and quantitative study of megakaryocytes.

Assuming an immune basis in ITP it is conceivable that since the platelets and the megakaryocytes have antigens in common, they are therefore both attacked (Humphrey 1955; Vaquez et al. 1960; van Boxtel 1972).

Antibodies have been demonstrated on megakaryocytes from some ITP patients and on normal megakaryocytes incubated with sera from patients with chronic ITP (Pisciotta et al. 1953; Mac Kenna 1962). The functional significance of these observations is not clear. In only very few of the many experimentally induced thrombocytopenias has an impairment of megakaryocyte function and platelet production been suggested (Witte 1955; Rolovic 1970a). A single patient with, probably, an immunologic pathogenesis of a reduced number of megakaryocytes has been described (Quattrin et al. 1963).

One would expect that in cases of drug induced immune thrombocytopenia megakaryocytes are also attacked. However a recent survey by Mc Vie (1973) gives no evidence of a disturbance of megakaryocyte function and platelet production in these cases. Based on a normal platelet life span or an inconsiderable decrease in platelet life span in some patients with a drug induced thrombocytopenia, Stijnen (1973) suggests the existence of an allergially impaired bone marrow function. However, to our opinion it is unlikely that in cases of a drug allergy platelets would be completely, or nearly completely, spared while megakaryocytes are extensively damaged.

1.2.3. Thrombocytopoiesis and megakaryocytopoiesis in conditions with abnormal platelet counts

The platelet generating capacity and platelet production rate can be studied in megakaryocytes. Not only the number of megakaryocytes, and the volume but also the ploidy, the nucleus-cytoplasm ratio, the amount of platelet generating cytoplasm and the maturation of the cytoplasm are decisive. Moreover it is necessary

to know the maturation rates of megakaryocytes and the velocity of platelet release. Information about the platelet volume gives insight into the amount of platelets which can be formed from the total megakaryocyte volume and into the degree of stimulation of thrombocytopoiesis. In man only some of the factors mentioned regarding megakaryocytopoiesis and thrombocytopoiesis are known.

The numbers of megakaryocytes in various conditions have been most extensively studied. An increased number of megakaryocytes has been reported in various conditions for instance during an increased platelet turnover or an increased pooling of platelets in the spleen, in conditions which may lead to thrombocytopenia, and in primary or secondary thrombocytosis. In table 1 the results of some studies in marrow biopsies are summarized. Maximal increases in the number of megakaryocytes, up to 10 times the normal values, were observed in cases of thrombocytosis and Di Guglielmo's syndrome. A decreased number of megakaryocytes has occasionally been reported in congenital disorders; amegakaryocytic thrombocytopenia (Shaw et al. 1959) and frequently secondary to bone marrow damage by drugs or radiation.

The diameter of megakaryocytes in various pathological conditions has been amply studied by Harker and Finch (1969). They found that megakaryocyte size was inversely related to the circulating platelet count. There are two exceptions: autonomous thrombocytosis and ineffective thrombocytopoiesis. In cases of autonomous thrombocytosis, as observed in myeloproliferative diseases (see section 1.2.1.), the mean volume of megakaryocytes is not decreased but on the contrary is sometimes even 2½ times more than normal (Harker et al. 1969; Kutti et al. 1973). In cases of ineffective thrombocytopenia the mean volume of megakaryocytes was decreased or normal, with mean values of about 50% of normal values, although there exists a thrombocytopenia.

An increased mean megakaryocytic size was demonstrated in hypoproliferative thrombocytopenias e.g. in patients treated with cytostatic drugs. The mean megakaryocytic diameter was also nearly always increased in thrombocytopenia caused by an increased platelet turnover. This resulted in an increase in the megakaryocytic volume of between 10 and 50% in idiopathic thrombocytopenic purpura, lupus erythematosus, chronic lymphatic leukaemia, lymphosarcoma, Hodgkin's disease and consumptive coagulopathy (Harker et al. 1969, 1970). In thrombocytopenia caused by an enlarged splenic pooling of platelets,e.g. in patients with congestive splenomegaly or osteomyelofibrosis, the mean megakaryocytic diameter was

Table 1

Quantitation of megakaryocytes in histological sections of bone marrow in various diseases

Studies	Megakaryocytes no. of sections (x normal)	Number of patients	Platelet count ($x10^3/\mu l$)	Diagnosis
Barta (1932)	0.8- 1.2	3	103- 138	typhoid fever
	1.7- 1.9	2	158- 186	sepsis
	1.4- 1.5	2	118- 137	acute leukaemia
	0.7- 0.9	3	104- 139	pernicious anaemia
	1.4- 1.6	2	242- 267	gastric cancer
Williams (1942)	0.3- 4.9	39	?	pneumonia
Franzén et al. (1961)	0.8- 5.8	20	60-1100	chron. myelogenous leukaemia
	0.4- 4.6	20	120- 960	polycythaemia vera
Harker and Finch (1969)	0.02-1.6*	15	2- 84	hypoplasia or marrow infiltration
	1.5-11.4*	8	14- 99	ineffective production (Di Guglielmo, B_{12} defic., familial thrombocytopenia)
	1.0- 3.1*	9	42- 86	splenomegaly (Hodgkin, myelofibrosis, congestive)
	1.1- 4.7*	7	4- 70	sec. immune thrombocytopenia
	2.0- 2.6*	4	9- 100	platelet consumption (coagulopathy)
	2.4- 8.5*	9	434-1484	reactive thrombocytosis
	1.3- 8.9*	7	265-2670	thrombocythaemia
	1.9- 8.0*	5	430-1440	polycythaemia vera
	3.6- 9.4*	3	507- 981	chron. myelogenous leukaemia
Kutti et al. (1973)	2.2- 8.1	20	148-1045	polycythaemia vera

*number of whole megakaryocytes, not the number of sections

normal or increased.

A decrease in the mean diameter of megakaryocytes was observed in patients with a reactive thrombocytosis e.g. caused by infectious diseases, iron deficiency or malignant diseases (Harker et al. 1969). In patients with chronic myeloid leukaemia with thrombocytosis a decreased megakaryocytic diameter was observed not only by Franzén et al. (1961) but also by Harker et al. (1969), suggesting not an autonomous but a reactive proliferation of megakaryocytes.

The total megakaryocyte volume, the product of the number of megakaryocytes and their volumes, was calculated by Harker and Finch (1969) and Kutti et al. (1973). In pathological conditions this sometimes proved to be 12 times the normal value. These maximal values were seen in patients with polycythaemia vera and idiopathic thrombocytosis.

The few investigations of maturation time of megakaryocytes in man showed no clear difference between normal and stimulated thrombocytopoiesis e.g. after massive blood loss (Krevans et al. 1955; Harker et al. 1969). However the number of observations is still scanty. The influence of a suppression of thrombocytopoiesis on the maturation time of megakaryocytes in man has not been examined.

No other observations have been reported concerning the influence of stimulation or suppression of thrombocytopoiesis on the nuclear-cytoplasm ratio and the platelet release in man.

Conclusion

Of the methods now available to characterize megakaryocytopoiesis quantitatively, the estimation of the total megakaryocyte volume per bone marrow volume or per kg body weight seems to be the most useful because of the good positive relationship between this value and the calculated platelet production.

This study will attempt to give a better insight into the megakaryocytopoiesis and thrombocytopoiesis of ITP, especially bone marrow function, by studying qualitative and quantitative aspects of megakaryocytes with methods which are relatively new in this field.

CHAPTER 2

OBSERVATIONS ON MEGAKARYOCYTES BY MORPHOMETRY AND BY PLOIDY QUANTITATION

2.1. INTRODUCTION

2.1.1. The number of megakaryocytes

The value of megakaryocyte counts will depend on the representativity of the marrow sample for the marrow as a whole. Therefore it is necessary to know:

1. if the marrow cellularity in different bones and in different localisations within a bone is constant and if there is a homogeneous distribution of various cell types.
2. if the marrow sample is representative of that marrow localisation of the body. This seems especially questionable in aspirated marrow samples.

There are only a very few accurate data concerning bone marrow cell constancy. Williams (1942) found a considerable variation in the cellularity of the marrow from different bones. So if megakaryocyte counts are expressed per unit marrow volume the marrow sample should always be taken from the same localisation. However, in different parts of a particular bone marrow localisation a constant cellularity has been reported by Barta (1932), Chan et al. (1966) and Krizsa et al. (1966). It is generally accepted that there is a uniform distribution of different cell types in different marrow localisations (Barta 1932; Custer and Krumbhaar 1936; Nickerson and Sunderland 1937; Williams 1942; Chan et al. 1966; Krizsa et al. 1966; Harker 1968a). The last mentioned author found a standard error of the mean of only 5% for the number of megakaryocytes in human marrow obtained from multiple biopsies of the right and left iliac crest.

Sometimes it is suggested that marrow aspirates are less representative for the marrow than marrow biopsies (Diggs 1948; Presley 1952). However this has never been proven by comparison of megakaryocyte counts in aspirates and biopsies from the

same marrow localization.

Counting megakaryocytes in bone marrow aspirates

Techniques for counting the number of megakaryocytes

Megakaryocyte counts in marrow aspirates can be carried out by a variety of techniques e.g. techniques using bone marrow smears, coverslip preparations (Diggs and Hewlett 1949), thick drop preparations (Osgood 1948) and haemocytometric methods (Pizzolato 1948). All methods using aspirated marrow have theoretical and practical disadvantages, namely:

1. The mentioned representativity of the aspirated marrow.
2. The dilution of marrow with aspirated blood is unknown and varies with every puncture. This variable dilution causes an important error in determining megakaryocytes per µl. This error can be overcome to some extent by determining megakaryocytes in proportion to nucleated red cells or young myeloid cells.
3. There is a variable loss of megakaryocytes during preparation. This can be concluded from the wide range of normal values found in different studies and from a comparison of megakaryocytic counts in aspirated marrow and marrow biopsies.
4. A number of unknown variables and errors are introduced: by taking a relatively small sample from a non homogeneous aspirate; by the settling out of cells; by the dilution error; and by the counting technique using a haemocytometer (Ebaugh, 1951).

In smears and coverslip preparations there is an irregular distribution of megakaryocytes. In smears the megakaryocytes tend to concentrate at the far end and at the lateral margins of the preparation. In the best coverslip preparations the distribution is still unequal (Diggs 1948). Counting of megakaryocytes in marrow aspirate suspensions with the aid of a haemocytometer (the chamber method) gives better results than megakaryocyte counts in smears, mainly due to the more even distribution of the megakaryocytes.

The results of megakaryocyte counts from smears, coverslip and thick drop techniques reported by Pizzolato (1946), Ebaugh (1951), Garg (1971) and Harker (1971) are very unsatisfactory. Normal megakaryocyte counts vary greatly in these studies and there is a wide range in each study; much more than the range found with methods using a haemocytometer or histological sections. For this reason Presley (1952) stopped the time consuming counting method in marrow smears and

used a screening method.

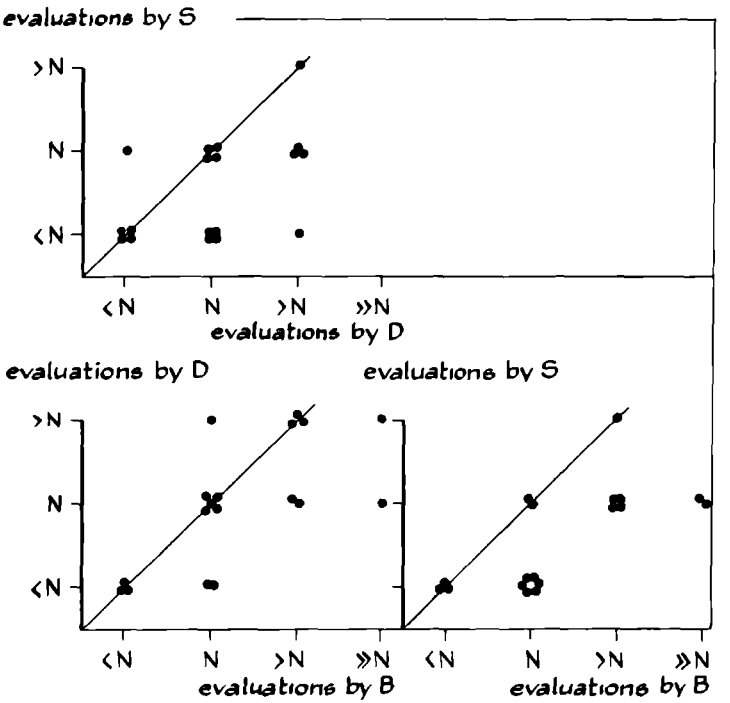

Fig. 2. Comparison of the evaluations of the number of megakaryocytes by screening in the same 18 bone marrow smears by 3 experienced workers (S,D and B). N:normal number of megakaryocytes; > , >> N: increased number; < N: decreased number

Pizzolato (1948) compared various chamber method studies and found that the results were in agreement with each other. The range for normal values in individual studies was often much smaller than with the other above mentioned methods which also employed aspirated material. Good results were obtained by Pizzolato (1948): the highest value for normal subjects was only about 2.5 times greater than the lowest value. The admixture of current blood cells in aspirated marrow

can be prevented by isolation of particles of bone marrow from aspirated material. One would expect that estimations of the number of megakaryocytes in dispersions of the disintegrated tissue would give better results. Japa (1943) used such a method but found too few megakaryocytes in normal subjects; 0.25% ± 0.08 (1SD) of the nucleated cells.

In conclusion, the results of methods for counting the number of megakaryocytes using aspirated fluid marrow samples are unsatisfactory, especially regarding the questionable representativity of the sample. Of these methods counting techniques employing a haemocytometer give the best results.

Counting of megakaryocytes in histological marrow sections

The use of bone marrow biopsies or particles of aspirated marrow (Lundin et al. 1972; Kutti et al. 1973), for the counting of megakaryocytes has the following advantages over the use of the previously discussed aspirated fluid marrow samples:

1. There is no loss of megakaryocytes during preparation.
2. The sample is more representative of the marrow as a whole.
3. There is no dilution with peripheral blood.
4. There are no faults introduced by preparation of the smears or dilutions.

Some disadvantages are the time consuming preparation, and the overestimation of the number of megakaryocytes. In a histological preparation only a portion of each megakaryocyte appears in a single section and a single megakaryocyte is therefore counted several times in serial sections. This "multiple counting" makes a correction of the number of cross-sections of megakaryocytes counted necessary. The theoretical number (N) of cross-sections of a megakaryocyte in histological sections is given by the formula (Harker 1968 a):

$$N = \frac{2R}{T} + 1 \qquad (1)$$

2 R is the true (maximal) diameter of the megakaryocyte

T is the thickness of the section

N is the number of cross-sections (cuts) per megakaryocyte

The diameter of the megakaryocyte in the prepared histological material is influenced by the nature of the fixation and embedding. The recognizability of cross-sections of megakaryocytes depends on the staining, the maturation stage of the cytoplasm and the presence or absence of a part of the typical nucleus. In practice these factors lead to a mean multiple megakaryocyte counting factor of about 7 in histological sections with a thickness of 3 µm, while the

multiple counting of smaller cells e.g. nucleated erythroid cells may be disregarded in the counting of these cells (Harker 1968a). Comparing the results of counting of megakaryocytes in different studies, the following points are to be taken into account:

1.The number of cross-sections of megakaryocytes depends not only from the number of megakaryocytes but also from the diameter of megakaryocytes.

2. The thickness of the histological section influences the number of cross-sections per megakaryocyte.

3. Shrinkage of the marrow during the histological preparation influences the number of cross-sections per unit marrow volume.

4. The recognizability of the cross-sections of megakaryocytes depends from staining techniques and thickness of the section.

5. The significance of the number of cross-sections of megakaryocytes per unit marrow volume depends on the cellularity of the examined marrow. There is a difference in cellularity between different bones.

All these factors can markedly influence the number of megakaryocyte cross-sections per unit marrow volume or per area of the histological section. If megakaryocyte numbers are given as a proportion of other marrow cells point 5 can be disregarded. So it has to be realized that the megakaryocyte numbers, especially when given per test volume, are only relative data.

Results of studies of the number of megakaryocytes in histological sections are summarized in table 2. Barta (1932) found in sections of 4-5 µm thickness, 50 to 60 megakaryocytes per 100 test fields in normal subjects. As he did not describe the dimensions of the examined test fields, his numbers are only of importance in showing the narrow range of his normal values. Dameshek (1935) reported differential counts in marrow sections. 0.7 -3.3% of the nucleated cells were megakaryocytes. The number of analyzed cells studied (300), was too limited since Custer and Krumbhaar (1936) have remarked the inaccuracy in a differential count of 500 cells for determining the incidence of cells as sparsely distributed as megakaryocytes. Based on empirical data they decided to count a total of about 30 megakaryocytes and found that 0.15-0.37% of the nucleated cells were megakaryocytes (table 2). Nickerson and Sunderland (1937) recommended examining still more nucleated cells and reported that 0.25 to 0.42% of the nucleated cells were megakaryocytes. In the studies of Custer and Krumbhaar (1936) and Nickerson and Sunderland (1937) material for normal controls was obtained by post mortem examinations, sometimes of patients diagnosed as purpura, polycythaemia vera, chronic haemorrhage, neutropenia, pulmonary embolism and some other abnormalities with known alterations in the number of megakaryocytes. One of the first reports

of numbers of megakaryocytes in presumable normal subjects is given by Williams

Table 2

The number of megakaryocytes in bone marrow sections in "normal*" subjects

Studies	Megakaryocyte count no. of sections				Thickness of section µm	No. of subjects studied
	% of nucleated cells		per mm^3 marrow			
	mean	S.D.**	mean	S.D.**		
Custer and Krumbhaar (1936)	0.25	24				8
Nickerson and Sunderland (1937)	0.33	17			6	9
Williams (1942)			2747	31	5	16
Franzén et al. (1961)			5087	33	6	20
Harker et al. (1969)	0.33	13			3	15
Lundin et al. (1972)			6300	42	3	25
This study			5495	28	5	14

* In some of these studies the examined subjects were suffering from diseases which may influence megakaryocytopoiesis

**SD: as % of mean value

(1942). He studied the vertebral marrow of 16 patients who died some hours after traumatic injuries, and reported 1791-4837 megakaryocytes per mm^3 of marrow (table 2). In a study of megakaryocyte counts in polycythaemia vera and chronic myelocytic leukaemia Franzén et al. (1961) used patients with Hodgkin's disease as control subjects. They counted 5087 megakaryocytes per mm^3 in the control subjects. Although in Hodgkin's disease the blood platelet count is frequently moderately increased (Abrahamsen 1970), this figure is in accordance with the finding of 6300 megakaryocytes per mm^3 in healthy control subjects by Lundin et al. (1972). Harker et al. (1969) reported in biopsies of normal subjects a nucleated red cell-megakaryocyte number ratio of 465(±)60:1. They were the first to correct the number of observed megakaryocytes for the multiple

counting of megakaryocytes brought about by the large diameter of megakaryocytes in comparison with the thickness of the histological section . Assuming 22% of the nucleated cells are nucleated red cells, one can calculate that Harker's findings correspond to a number of sections of megakaryocytes totalling 0.33% of all the nucleated cells.

Conclusion

Although the described studies often show differences in examined bone marrow localizations, histological techniques, thickness of sections and selection of test areas, the similarity of the megakaryocyte counts in control subjects and the small range in most studies supports the validity of the method. The range for normal values in histological sections of biopsies is smaller than the range in most studies concerning megakaryocyte numbers in aspirated marrow, which indicates that estimating the number of megakaryocytes in histological sections in the more accurate method.

Total number of megakaryocytes in the human body

The number of megakaryocytes in the body has not been studied by examination of post mortem material but only employing isotope dilution methods. A marrow biopsy is taken some time after the injection of radioactive iron. In the marrow sample the ratio number of megakaryocytes/ number of nucleated red cells is estimated. Using plasma radioactive iron turnover figures, the total number of nucleated red cells in the body is estimated. With this knowledge and the ratio megakaryocytes / nucleated red cells in the marrow sample, the total number of megakaryocytes in the body can be calculated (Harker 1968a). A variant of this principle was used by Fillet (1971), measuring the ratio: number of megakaryocytes /radioiron activity in the marrow sample. A disadvantage of the present isotope dilution methods is the radiation exposure of the patient. Moreover methods using ^{59}Fe can only be applied in subjects with normal erythropoiesis and iron kinetics. Harker's (1968a) results with this method showed a small range for normal subjects, $6.1 \pm 0.7 \times 10^6$ megakaryocytes/kg body weight. However when there is a normal erythropoiesis one can expect that the erythroid mass/kg body weight will be constant. In this case the use of the isotope dilution method has no advantages over the estimation of the number of megakaryocytes in the marrow sample as a proportion of the nucleated red cells. On the other hand in cases of anaemia, the isotope dilution method has the advantage of correcting the megakaryocyte count in histological sections for the eventually abnor-

mal marrow erythroid mass/kg body weight. But especially in cases of anaemia the risk of abnormal iron kinetics often makes this method impracticable. So in clinical practice isotope dilution methods, using iron kinetics to estimate the total number of megakaryocytes in the body, has often no advantage over the estimation of the number of megakaryocytes as a percentage of nucleated red cells in a biopsy.

Conclusions

The best results in estimation of the number of megakaryocytes have been obtained in marrow samples from marrow biopsies. As the diameters of megakaryocytes are more than the thickness of histological sections one has to take into account a multiple counting of megakaryocytes because one megakaryocyte is visible in more than one histological section. When there is a normal turnover of erythroid or myeloid cells, comparison of the number of megakaryocytes with the number of these haemopoietic cells in marrow biopsies gives good information regarding the number of megakaryocytes, not only in the histological section, but also in the whole body.

2.1.2. Megakaryocyte volume and cytoplasm-nucleus ratio

The importance of the volume of megakaryocytes for the platelet production capacity is often underestimated. Many studies concerning alterations in thrombocytopoiesis have only been concerned with the number of megakaryocytes. One has to realise that a relatively small increase in the diameter of a sphere results in a rather large increase in the volume.

The size of megakaryocytes increases both with increases in ploidy level and with degree of cytoplasmic differentiation (Japa]943;Harker 1968a;Ebbe et al. 1968a; Odell et al. 1970; Stadhouders 1974). Therefore the mean megakaryocytic volume will depend on the frequency distribution of ploidy levels and maturation stages. These depend on the degree of stimulation or suppression of megakaryocytopoiesis. In animals Stadhouders (1974) found a fixed cytoplasm-nucleus volume ratio for a particular maturation stage which was defined by the number of α granules. In Guinea pigs this ratio was about 2 for very immature and about 8 for mature megakaryocytes. This implies that the volume of a megakaryocyte of a particular ploidy level at the end of maturation is 3 times more than at the start of maturation. This constancy of the cytoplasm-nucleus volume ratio implies

that the megakaryocytic volume of a 64 N megakaryocyte will be twice the volume of a 32 N megakaryocyte of the same maturation stage. So there is an overlap of volumes of mature megakaryocytes at a particular ploidy level and immature megakaryocytes from a subsequent ploidy level.

Harker and Finch (1969) found a constant mean cytoplasm-nucleus volume ratio of megakaryocytes in normal human subjects, in patients with idiopathic thrombocytopenic purpura, in hypoplastic thrombocytopenia and also in B_{12} deficiency-thrombocytopenia, although in the last case there was a decreased platelet production per nuclear unit. In the first stages of acute changes in megakaryocytopoiesis in rats Harker (1968a) found a small decrease in the mean cytoplasm-nucleus ratio. This decrease can be explained by an increase in the number of immature megakaryocytes.

Influence of the technique employed for measurement of megakaryocytic size

Megakaryocytic size is measured by estimation of the diameter or the surface area of the image of intact megakaryocytes in smears or squash preparations or the diameter of sections of megakaryocytes in histological sections. Comparing the results of measurements of megakaryocytic size one has to take into account:
a. the mode of preparation of the marrow sample
b. whether the measurements were done in marrow smears or in sections
c. in random sections or serial sections.

a. Marrow sample preparation
Estimation of megakaryocytic diameter is usually done on fixed and stained marrow samples. Fixation, dehydration, embedding and staining often cause shrinkage or swelling of the various bone marrow cells (Harker 1968a). Diameter measurements therefore are only relative and does not represent the diameter in vivo. Therefore measurements of megakaryocytic diameter are valid for comparative studies using the same method, but large differences can be expected between studies using different methods of marrow preparation.

b. Measurements in smears or in sections
In smears and squash preparations of marrow particles the megakaryocytes are more or less flattened. The degree of flattening is different for the various megakaryocytic sizes (Odell et al. 1970). As a consequence of these differences in thickness of megakaryocytes of different volumes in smears and squash prepa-

rations, measurements of the megakaryocytic diameter (which vary from 30 to 60 µm), or the surface area of the image of megakaryocytes cannot be used for calculations of the megakaryocytic volume.

c. Histological studies of serial or random sections

In serial section studies megakaryocytes appear as approximately spheroidal cells (Harker 1968a). As the diameters of megakaryocytes are larger than the thickness of the histological section one has to measure the diameter of a section passing through the centre of the megakaryocyte. If serial sections of each megakaryocyte are not available one can use the known relationship between the mean maximal diameter of spheres of different sizes and the diameter of sections through the spheres; given by the formula (Underwood, 1970):

$$\bar{r} = \frac{\pi}{4\,\bar{m}} \qquad (2)$$

$\bar{r}$ is the mean maximal radius of the spheres

$\bar{m}$ is the mean of the reciprocal values of diameters of sections of the spheres

This formula assumes infinitely thin sections and the recognition of all small sections of a sphere. However in practice this formula's use is restricted because

a. histological sections through megakaryocytes have a certain thickness and

b. not all sections of megakaryocytes are recognized.

Regarding a., therefore, the relationship between the maximal diameter of a single megakaryocyte and the mean diameter of all the sections through that megakaryocyte depends on the relationship between the maximal diameter and the thickness of the sections. When the thickness of the section is very large with respect to the maximal diameter of the megakaryocyte the diameter of a section of a megakaryocyte will be the true diameter of that megakaryocyte. With decreasing thickness of the section the difference between the maximal diameter and the mean diameters of sections of megakaryocytes will increase. If the size of the megakaryocyte decreases and the thickness of the section is constant then the difference between the maximal diameter and the mean diameter of the sections will decrease.

Regarding b it is easier to recognize small sections of megakaryocytes in serial section studies than in random studies, e.g. with the aid of photomicrographs of adjacent sections. Recognizing more small sections results in a smaller mean diameter of the sections of the megakaryocytes. So the difference between the maximal diameter and the mean diameter of the sections will be greater

in serial sections than in random sections.

From the foregoing it must be concluded that the results of megakaryocytic size measurements in histological sections are only relative.

Results of measurements of megakaryocytic size

With a photomicrogravimetric technique Ebbe et al. (1968a) measured the surface area of the image of megakaryocytes of rats in smears. The mean surface areas of successive maturation stages, of megakaryocytes types I, II and III, were respectively 771, 1401 and 1799 μm^2. Using the same technique Odell et al. (1970) found about the same ratio for these different maturation stages in squash preparations. The ratio between the surface area of 8N, 16N and 32N type I megakaryocytes was 10:17:26. With a microplanimetric procedure Albrecht et al. (1974) found that the surface area of the image of normal human megakaryocytes in smears was often between 500 and 3000 μm^2. Franzén et al. (1961) illustrated the above mentioned influence of the flattening of human megakaryocytes in smears. They found a larger surface area of the image of megakaryocytes in smears than in histological sections. In patients with Hodgkin's disease Franzén et al. (1961) reported a cross section area of megakaryocytes between 140 and 800 μm^2, with a peak value at 350 μm^2. Despite the differences in the methods used there is rather good agreement with the results of studies concerning the size of sections of megakaryocytes in histological sections. Lundin et al. (1972) found a mean cross section area in megakaryocytes of 296 $\mu m^2 \pm 60$ in normal man. Exact comparison of the mean diameter of megakaryocytes with the mean surface area of the images of megakaryocytes is not possible without some knowledge of the frequency distribution of both. The calculated surface area of the images of megakaryocytes, using the mean diameter of spheres of different size, is much less than the true mean surface area of images of megakaryocytes. Still the results of measurements of cross section areas of megakaryocytes of Franzén et al. (1961) and Kutti et al. (1973) agree with the measurements of diameters of sections of megakaryocytes made by Pennington (1970) and Harker et al. (1968a, 1969). Pennington (1970) found section diameters of 10 to 26 µm in random sections of megakaryocytes from rats. Harker et al (1968a, 1969) found no differences between the size of megakaryocytes in man and rats and calculated the mean true diameter of human megakaryocytes as 20.8 µm $\pm$ 3.3 from measurement of diameters in random section studies corrected with the quotient of the true diameter and mean diameter of the sections of megakaryocytes found in serial section studies.

Calculations of mean megakaryocytic volume

In principle calculation of mean megakaryocytic volume can be done using different data:

1. Frequency distribution of individual volumes of megakaryocytes.
2. Frequency distribution of individual true diameters of megakaryocytes
3. Total megakaryocytic volume or total megakaryocytic surface and the number of megakaryocytes.

Up to the present such data are almost lacking and no exact data on mean megakaryocytic volumes are available. Harker (1968a) calculated a mean megakaryocytic volume of 4700 $\mu m^3 \pm$ 100, from the mean megakaryocytic diameter, with the formula:

$$\text{volume of a sphere} = 0.5236(2R)^3 \qquad (3)$$

R is the radius of a sphere

However the volume calculated from the mean diameter of spheres of different sizes is less than the mean volume of the spheres.

Conclusions

There are no exact measurements of megakaryocytic volume available. Upto now the best available approaches are measurements of the true diameters of megakaryocytes in histological serial section studies. This has yielded diameters varying from about 14 to 28 µm (Harker 1968a, 1969). These data are probably relative as a consequence of the influence of the histological preparation of megakaryocytes on their size.

2.1.3. Total megakaryocyte volume

As mentioned in chapter I the total megakaryocyte volume of the marrow (the product of the number and volume of megakaryocytes) indicates the potential platelet production capacity of the marrow if there is a steady state with normal maturation time of the megakaryocytes, a normal cytoplasm-nucleus ratio, a normal volume of delivered platelets and when there are no extramedullary platelet producing megakaryocytes. In studies of megakaryocytopoiesis attention has often only been paid to the number of megakaryocytes. Often the large effect of a small increase in the diameter of the megakaryocytes on the platelet production capacity is neglected. For instance, one can calculate that Pennington's (1971b) reported small difference in polyploidy distribution of megakaryocytes, which

means a small difference in the diameter of megakaryocytes between normal and ITP patients, implies a two fold increase in the total megakaryocyte volume in ITP patients (table 3). In the studies by Lundin et al. (1972) and Kutti et al. (1973) the mean megakaryocyte area of sections of megakaryocytes was measured by planimetry and the total volume of sections of megakaryocytes in marrow sections was determined by calculating the product of the mean megakaryocyte area times the number of sections of megakaryocytes per mm^2 bone marrow. This resulted in a total megakaryocyte volume of 0.57% of the marrow volume in normal subjects. Until now only Harker et al. (1968a, 1969) has tried to calculate the total megakaryocyte volume of the marrow in the body. As already stated they estimated the total number of megakaryocytes in the body ($6x10^6$/kg body weight) with a sensitive isotope dilution method and calculated the mean volume of megakaryocytes (4700 μ^3) from the mean diameter of megakaryocytes in marrow biopsies. Regardless of some imperfections in the methods used, Harker found a positive correlation between the total megakaryocyte volume and the effective platelet production. As, in theory, the platelet generating capacity of the bone marrow will depend on the number of megakaryocytes and their individual volumes, and a close relationship was found (Harker et al. 1969) between the total megakaryocyte volume of the marrow and platelet production, we tried to obtain information about the number of megakaryocytes, together with their volumes, in normal subjects and ITP patients. For this reason we studied the total megakaryocyte volume of tissue sections of marrow biopsies with a morphometrical method which is described in 2.2.1.

2.1.4. Megakaryocyte ploidy

Techniques for determination of megakaryocyte-ploidy

Ploidy determinations are approached in three ways:

a. the number of "nuclei" or lobes of nuclei, (Japa 1943, Harker 1968b) may be counted
b. DNA may be quantitated
c. the nuclear volume may be measured

a. The number of "nuclei" or lobes of the nuclei. As discussed in chapter I the nuclear segmentation will be visible by light microscopy if the polyploidization of the individual megakaryocyte is completed. Therefore there will be no good

relationship between the number of nuclear lobes and the DNA content in immature megakaryocytes. Moreover the technique of lobe counting is not free from criticism (de Leval et al. 1971).

b. The DNA content

DNA measurements of individual Feulgen stained nuclei of megakaryocytes has been conducted by absorption-cytophotometry and microfluorometry (Paulus et al. 1971). Microfluorometry with incident light proved to be superior (Böhm et al. 1968). The measured stain content is related to the amount of DNA present. In recent studies some influence of the structure of the chromatin network on the Feulgen-DNA values was observed. Nuclei with a condensed chromatin showed lower Feulgen-DNA values than nuclei with the same karyotype in a similar phase of the generation cycle but which had a looser chromatin meshwork (James 1973). The chromatin structure of megakaryocytes is not constant. This, in part, explains the large number of megakaryocytes with ploidy values (N) other than 8 N, 16 N, 32 N and 64 N, suggesting DNA synthesis, in comparison with the number of megakaryocytes synthesising DNA according to thymidine labelling in autoradiographic studies.

c. The nuclear volume

A positive relationship between the nuclear volume and the DNA content of megakaryocytes has been observed by Queisser et al. (1971). If there was a good relationship between nuclear volume and ploidy level then we might expect the nuclear volume would increase in discrete twofold steps. However results of more exact measurements of nuclear volumes carried out by Stadhouders (1974) showed a large number of megakaryocytes which fell in between the expected size classes. This suggests that the relationship between nuclear volume and the DNA content of megakaryocytes is not very constant and therefore determination of nuclear volume is not a reliable index for ploidy level.

In all studies concerning ploidy distribution of megakaryocytes one has to realise that there is an unavoidable error resulting from the sampling of a small number of megakaryocytes from a large population (Paulus et al. 1971). This sampling error is important in all types of studies as the number of examined megakaryocytes is rarely more than 100.

In summary it has to be concluded that the methods employed up to now for determination of the ploidy distribution of megakaryocytes have been rather rough ones.

Results of determinations of megakaryocyte ploidy

In normal human subjects the ploidy values of megakaryocytes varied between 4N and 64N, with a frequency peak of about 50 percent at the 16N level. There are more 32N megakaryocytes than 8N megakaryocytes (see table 3). There are only a few studies of ploidy values under pathological conditions available (Undritz et al. 1969; Lagerlof 1971; Queisser et al. 1971, 1973; Kinet-Denoel et al. 1973) In some patients with polycythaemia vera, chronic myeloid leukaemia, acute leukaemia and preleukaemia a decrease in ploidy values has been observed. In chronic myeloid leukaemia even mature diploid megakaryocytes have been reported. Ploidy values of 128N have occasionally been seen.

Table 3

Polyploidy of megakaryocytes in normal subjects and patients with ITP

Study	No. studied		Megakaryocytes (%)				Conclusion concerning ploidy in ITP
	Norm.	ITP	8N	16N	32N	64N	
Japa (1943)	10		26	54	19	1	
		-					
Harker et al. (1969,1970c)	15		10	65	25		"megakaryocyte volume
		16					and nuclear lobe number increased"
de Leval (1968)	1		10	54	29	7	
		-					
Queisser et al. (1971)	3		14	63	23	-	"megakaryopoiesis
		3	16	57	27	-	unaltered"
Pennington et al.(1971b)	1		9	55	34	2	"very similar"
		3	13	40	35	11	
Kinet-Denoel et al. (1971)	4		17	46	29	7	8 out of 10 patients·
and		5	47	26	20	6	"marked shift to-
Paulus et al. (1973)		10					ward lower ploidy"
Lagerlöf (1972)	3			max			
This study	14		15	41	37	7	Shift toward
		14	8	37	48	7	higher ploidy

Results of ploidy measurements in patients with ITP are very contradictory (table 3). Harker et al. (1969, 1970c) employing cell and nuclear size measurements together with lobe counting, found an increase in megakaryocyte size and ploidy in 16 patients with ITP. In the studies of Queisser et al. (1971) and Pennington et al. (1971b), each concerning 3 patients with ITP a normal ploidy distribution was reported. However, if one calculates from the data of Pennington the mean ploidy value in his patients with ITP, this seems to be 19% more than normal. In studies from the group of Kinet-Denoel and Paulus 8 out of 10 patients with ITP showed a decrease in ploidy values, with a frequency peak at the 8N level in several patients (Kinet-Denoel et al. 1971, 1973; Paulus et al. 1973).

In induced immune thrombocytopenia in animals an increase in polyploidy of megakaryocytes has been described (Penington et al. 1970).

2.2. INVESTIGATIONS ON MEGAKARYOCYTES BY MORPHOMETRY AND BY PLOIDY QUANTITATION

2.2.1. Materials and methods

Platelet counts

Four ml blood samples were collected in disposable collecting tubes containing 4 mg di-K EDTA. Platelets were counted electronically after separation by means of differential centrifugation, essentially according to the method of Nakeff and Ingram (1970). Exactly 10 µl blood plus approximately 5 µl silicone oil (S.G. 1.040) was sucked up in a capillary micropipette. The pipettes were closed with Seal Ease (Clay Adams) and centrifuged in a microhaematocrite centrifuge (Hawksley) for 12 sec. at 18000 rpm. The platelet layer containing part of the micropipette was broken off at the oil level which, after centrifugation, remains above the red cell mass. Platelets were resuspended in 10 ml isotonic saline and counted with a Coulter Counter B equipped with a 50 µ tube. The counting procedure was carried out with settings: Amplification ½, aperture current ¼, lower threshold 10, upper threshold 96, Matching switch H 64, gain control 90, "separate locked" switch on "separate" and volume on 0.05ml. The mean platelet count for ages from 18 to 48 years was 207,700 ± 49,600 (1 SD) per µl blood.

Platelet survival

Platelet survival studies were done using tritiated diisopropylfluorphosphate (^{3}H-DFP) according to the method of Bithell et al. (1967).

In some patients with a severe thrombocytopenia, platelet survival studies were carried out with homologous ^{51}Cr labelled platelets, according to the method described by de Maat (1969). Circulating platelet-bound radioactivity was determined using daily blood samples.

Platelet half-life time was employed as the measure of platelet survival.

Bone marrow biopsy and histological preparation

Biopsy technique

Marrow biopsies were obtained from the posterior iliac crest under local anaesthesia with a needle as described by Jamshidi (1971). The specimens were approximately 2 mm in diameter and ranged from about 5 to 30mm in length upwards.

Histological preparation of biopsies

Fixation

Biopsy specimens were fixed for six hours in the dark in a freshly prepared mixture of Zenker's solution (Romeis 1968), formaldehyde and glacial-acetic-acid, (20:1:1). After fixation the specimens were rinsed for 24 hours with running tap-water.

Dehydration

Dehydration was performed sequentially with ethanol 70%, ethanol 96% and ethanol 100%, each for 1 hour . When the embedding procedure could not take place at once the specimens were stored in ethanol 70%.

Embedding

After 1 hour in benzoic acid methyl ester the specimens were placed in an embedding solution, a mixture of 99 parts methyl-methacrylate (BDH chemicals) and 1 part benzoic peroxide, for 24 hours at room temperature. After that the embedding solution was replaced by 9 parts of the mentioned embedding solution mixed with 1 part Plastoid-N (Röhm and Haas).Heat polymerization was performed in enclosed tubes at 50^{o}C for about 24 hours.

Tissue sectioning

Sections of 5µm thickness were cut.* Of each 5 successive sections only the first was stained and examined. Besides these random sections, one serial section study of an ITP patient was made.

*Microtome Leitz

Staining

A variant of Goldner's staining technique (Romeis 1968) was used according to the following procedure:

distilled water, 5 min.

haematoxylin Weigert solution*, 20 min.

rinse with tap water, 20 min.

rinse with distilled water, 1 min.

Ponceau-fuchsin-azophloxine solution*, 5 min.

acetic acid 1%, 15 sec.

phosphotungstic acid-orange II solution *, 5 min.

acetic acid 1%, 15 sec.

light green solution*, 5 min.

acetic acid 1%, 1 min.,repeated 3 times

ethanol 30%, 1 min.

ethanol 70%, 1 min.

ethanol 99%, 1 min.

*The solutions were prepared according to Romeis (1968). The dyes were obtained from Merck.

Morphometrical methods

Quantitation of megakaryocytes in histological sections of bone marrow biopsies was performed with a)stereologic methods using an ocular grid, b)methods using an ocular micrometer and c) photomicrography.

a) Stereologic methods, using an ocular grid

Morphometry, a term orginally used by geographers to indicate quantitative descriptions of geographical features, has been recently introduced into the field of microscopic anatomy. In biological morphology, morphometric data can be obtained by various means. When stereological methods are employed the procedures are often efficient and accurate (Weibel and Elias 1967). Stereology is a body of procedures, mainly geometrical-statistical, which may be used to obtain information about three-dimensional objects from two-dimensional images. This method was applied for the first time in the field of astronomy.

In 1847 the French geologist Delesse described the fundamental relationship of stereology, which states that in random sections of a testvolume the mean

fractional area of a testarea occupied by cross-sections of a component i, A_{Ai}, corresponds with the fractional volume of a component i, V_{Vi}, of the test volume

$$V_{Vi} = A_{Ai} \qquad (4)$$

In 1933 Glagoleff developed the point counting method to estimate the relationship between two surface areas. So planimetry could be done by superimposing a regular point lattice on the section and counting the points which lay on cross-sections of structures (fig. 3). So the fraction of points, P_{Pi}, laying on cross-sections of objects i, led to an estimate of volume fraction V_{Vi}, if the sections are 2 dimensional, ($V_{Vi} = P_{Pi}$). By the point counting procedure the measurement of the collective volume of objects included in the unit test volume is reduced to counting.

The number of objects

In counting objects in pictures of transparent sections the section thickness is not negligible. It is impossible to count the number of objects;one can only count the number of cross-sections of objects. The relationship between the number of objects, included in the unit test volume, and the number of cross-sections of objects depends on the relation between thickness of the section and the size of the objects. If the objects are spheres this relation will be (see:formula 1 and Harker 1968a):

$$N = N' : \left(\frac{2\bar{R}}{T} + 1\right) \qquad (5)$$

Where N is the actual number of objects per unit test volume.

N' is the number of cross-sections (cuts) of objects per unit test volume.

$\bar{R}$ is the mean radius of the spheres (not the mean radius of cross-sections of objects).

T is the thickness of the section.

It will be clear that without knowledge of the mean diameter of the spheres it is impossible to get information about the number of spheres included in the unit test volume.

A regular pattern of points and test lines, superimposed on the histological section image, was obtained by placing a test system, a Merz-(1967) grid, in one ocular of the microscope (fig.3). This grid contains a square with 36 test points (points of intersection of the curved test lines and the short horizontal lines). The length of the sides of the square (the test area) projected on the field of observation was calibrated as 189 µm. Measurements were performed at a magnification of 560 x.

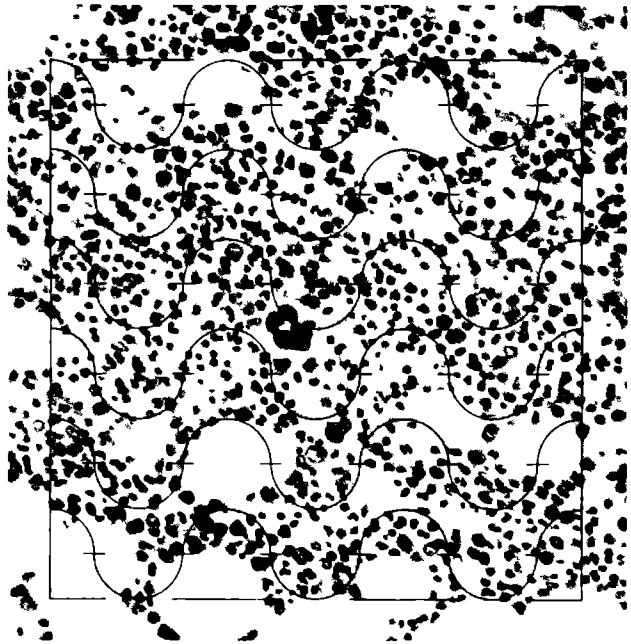

Fig.3.Histological section of a bone marrow biopsy with a superimposed ocular (Merz) Grid. The square with 36 test points was employed for determination of total megakaryocyte volume in histological sections.

This system fulfilled the conditions:a) rare components, as megakaryocytes,remained seldom undetected, b) as a rule no more than one test-point fell on the same cross-section of a megakaryocyte (Weibel, 1973).

Measurement procedure

Whole adjacent fiels free of bone trabeculae were examined regardless of their cellularity. The number of test points on megakaryocytes and the number of transsections of megakaryocytes per test area were counted. Megakaryocytes lying on the edge of the left and upper margin of the field were not counted. In most cases the number of test points on fat vacuoles, on sinusoids, on vessels, on nuclei of non-megakaryocytic haemopoietic cells, on ruptures in histological sections and the number of test line intersections with megakaryocytes were also determined. For statistical reasons a minimum of 100 fields was examined and, where necessary, more fields were examined until at least 50 points on megakaryocytes had been counted. One field corresponds to an area

of 189 x 189 μm^2 and with a volume of 179000 μm^3 fixed solid marrow since the sections were of 5 µm thickness.

b) Ocular micrometry

Measurements of diameters were carried out on a minimum of 100 cross-sections of megakaryocytes in histological sections employing an ocular micrometer, a 100 x objective and an ocular of 10 x. On the ocular micrometer each calibration line represented 0.8 µm and measurements were made to the nearest 0.8 µm. sections of megakaryocytes were measured in adjacent fields. Sections of megakaryocytes less than 10 µm in diameter could rarely be recognized with certainty. Megakaryocyte diameters were measured in one direction since the embedding procedure, using methyl-methacrylate, prevented distortion of cells by sectioning on the microtome. The measurements were done on projected images from sections of finite thickness. So the problems described above such as the Holmes effect, non-identifiable small transsections of megakaryocytes and overlap of opaque structures, also arise in ocular micrometry and in measurements on photomicrographs.

c) Photomicrography

The diameter of all cross-sections of each megakaryocyte was obtained by linear measurements on serial section photomicrographs of 101 megakaryocytes. Measurements were made with a simple ruler to the nearest 1 mm. The total magnification was 282 x. Moreover the number of transsections of each megakaryocyte were counted.

Expression of results of morphometrical determinations

Total megakaryocyte volume unccorrected for cellularity

This term is used for the volume density of megakaryocytes, the fractional volume of the bone marrow volume occupied by the collective volume of megakaryocytes. This is estimated by determining the relative area of a test area occupied by the collective area of cross-sections of megakaryocytes by use of the equation:

$$\frac{\bar{P}_{mkc}}{P_T} \times 100\ \% \qquad (6)$$

$\bar{P}_{mkc}$ is the mean number of test points on megakaryocytes per test area

P_T is the total number of test points (36) per test area.

In subjects with a normal erythropoiesis and myelopoiesis rather small ranges for the peripheral blood cell concentration, life span and production rate have been found. This is in accordance with the observed narrow range for the number

of myeloid- and erythroid cells per kg body weight. It implies that if there is a difference in the concentration of haematopoietic cells per bone marrow volume unit between individual subjects one can also expect a difference in the total marrow volume per kg body weight between individual subjects. For this reason the cellularity of the histological sections of bone marrow tissue has also been determined.

Cellularity of histological sections

The cellularity is expressed as the fractional volume of the bone marrow volume occupied by the collective volume of nuclei of haemopoietic cells (excluding megakaryocytes). This is estimated by use of the following equation:

$$\frac{\bar{P}_n}{P_T} \times 100\% \qquad (7)$$

$\bar{P}_n$ is the mean number of test points on nuclei of haemopoietic cells (excluding megakaryocytes) per test area. (Because nuclei were better visible they were prefered to whole cells as a parameter for cellularity).

Total megakaryocyte volume corrected for cellularity.

This term is used for the collective volume of megakaryocytes expressed as a percentage of the collective volume of nuclei of haemopoietic cells (excluding megakaryocytes). This is estimated by:

$$\frac{\bar{P}_{mkc}}{\bar{P}_n} \times 100\% \qquad (8)$$

Three parameters for megakaryocytic size are used:

a. True diameter

In histological sections the true diameter of megakaryocytes can only be estimated in serial section studies by measuring the diameter of the largest cross-section of all the sections through an individual megakaryocyte.

b. Mean diameter of sections of megakaryocytes

Henceforth this term will be employed for the diameter of random sections of megakaryocytes unless serial sections are expressly mentioned.

c. Mean area of sections of megakaryocytes

This is estimated by:

$$\frac{\bar{P}_{mkc} \times (189\ \mu m)^2}{\bar{N}_{mkc} \times P_T} \qquad (9)$$

$\bar{N}_{mkc}$ is the mean number of sections of megakaryocytes per test area.

$(189\ \mu m)^2$ is the surface of the test area.

The number of sections of megakaryocytes

a. Number of sections uncorrected for cellularity

This term is used for the mean number of sections of megakaryocytes per test area of $(189\ \mu m)^2$.

b. Number of sections corrected for cellularity

This term is used for the number of sections of megakaryocytes expressed per collective volume of nuclei of haemopoietic cells (excluding megakaryocytes). This is given by:

$$\frac{N_{mkc}}{P_n} \qquad (10)$$

Method for determination of megakaryocyte ploidy

Preparation and staining of cells

The aspirated marrow was collected in plastic tubes with an equal volume of anticoagulans*. Marrow smears were made, dried for 1 or 2 hours and fixed for 1 hour. The fixative contained 85% methanol (100%), 10% formalin, and 5% glacial acetic acid. Fixation was followed by a 10 min. rinse in distilled water. Fixed smears, enclosed in plastic, were kept in the refrigerator at $-20^{\circ}C$ until they were stained.

Feulgen reaction:

Hydrolysis was performed in 4N HCL at $28^{\circ}C$ in a thermostatically controlled water bath ($\pm 0.2^{\circ}C$) for 100 min. Smears were then kept in the staining bath with Schiff reagent for 1 hour at room temperature. The Schiff reagent was prepared according to the method of Graumann (1953) with the modification that 0.1 g pararosaniline instead of 0.5 g in 15 ml 1N HCL was used. The stained smears were twice placed in a freshly prepared sulphite solution for 15 min. The sulphurous acid solution was a mixture containing 5ml 1N HCL, 5 ml 10% $K_2S_2O_5$ and 90 ml distilled water. After rinsing with this sulphite solution the smears were washed with distilled water for 10 min, dehydrated for 30 min. in

*Composition of anticoagulans:
1 volume acid citrate solution (tri-Na-citrate 22 g, acidum citricum 8 g, dextrose anhydricum 25 g, distilled water 1000 ml)
4 volumes NaCl 0.85%
1 volume buffer (0.15M Na_2HPO_4, 0.15 M NaH_2PO_4) adjusted to pH 7.0 with 2.5N NaOH diluted with distilled water to an end concentration of 300m.osmol/l. This buffered acid citrate solution was filtered through a millipore filter of 0.45 µm pore size and wet sterilized at $100^{\circ}C$ for 30 min.

ethanol 70%, 30 min. in ethanol 96%, 30 min. in absolute ethanol and 5 min in xylol, mounted in Fluormount (Gurr) and left in the dark until measured.

Instrumentation

A microscope photometer (Carl Zeiss) with adjustable measuring diaphragm (MPV) and an optical arrangement that allowed successive illumination with transmitted and incident light,was used. The characteristic feature of the instrument is the optimum separation of the excitation beam from the fluorescence light emitted by the Feulgen stained nucleus. Light was provided by a stabilized tungsten lamp, 60 Watt, 12 V. The selection of the activating wavelength was obtained by an FL 546 and KP 630 (Schott) filter in the excitation beam and the dichroic mirror, FL 580, in the vertical illuminator. A barrier filter 630 was inserted in the emitted light path. Premature excitation during optical focusing of the cells was prevented by restricting the amount of transmitted light, especially light of the wavelength used for excitation of the Feulgen stained nuclei. The smears were screened using a 10 x objective. The cells were measured with a neofluar, Ph 100 x 1.30, oil immersion objective.The detector was a photometer with a variable measuring diaphragm in conjunction with a photomultiplier (Hamamatsu, HTV 446) with a red sensitive S-20 photocathode, type 9558A, which takes its extraction voltage from a stabilized high voltage supply (Knott, type NSPM-BN-600). Readings were performed with an amperometer (Kipp, Flatbed Recorder B.D.8).

Measurement procedure

Smears were screenedfor megakaryocytes with a low power magnification and transmitted illumination. A single cell was brought into the measuring field and the variable diaphragm was adjusted to the size of the cell. After focusing with an oil immersion 100 x objective the cells were excited by the incident light beam to a red fluorescence. The emitted fluorescent light was measured in arbitrary light units. Metamyelocytes in the environment of megakaryocytes were measured as reference diploid cells. To prevent selection of megakaryocytes all megakaryocytes in each smear were measured. At least 100 megakaryocytes were measured.

Expression of results of ploidy determination

Ploidy value is obtained by dividing the measured emission units of megakaryocytes by one half of the emission units of 2N, diploid cells such as metamyelo-

cytes. Megakaryocytes were compared with metamyelocytes from the same slide because there was a variation in the emitted light from metamyelocytes in different slides even when the slides were stained together. The ploidy histogram was plotted on semilogarithmic coordinates assuming a log normal ploidy distribution in each ploidy class. The limit between two successive ploidy classes is 1.416 (second root of 2) x ploidy of the lowest class. Recognition of 4N megakaryocytes is difficult and other marrow cells can also be tetraploid in the premitotic phase. For these reasons cells having a ploidy value less than 5.6 N (midpoint between 4N and 8N classes) were excluded. Beside the ploidy histogram was made, the mean ploidy value of all megakaryocytes in each subject was calculated.

Subjects studied by marrow biopsy for morphometry

Normal controls (14)

Control subjects were persons without known blood disorders and a normal blood platelet count , haemoglobin concentration and leucocyte count. They were not suffering from diseases with known altered megakaryocyto-or thrombocytokinetics. In most cases the bone marrow biopsy was done to exclude metabolic bone diseases (table 4).

Thrombocytopenia was defined as a platelet count less than 110,000/µl, (the mean platelet count of normal subjects 208,000/µl, minus twice the standard deviation, 100,000/µl).

Patients with (a history of) chronic ITP (18). Chronic idiopathic thrombocytopenic purpura (ITP) was diagnosed if the following three criteria were met:

1. there was a thrombocytopenia for more than 3 months (or patients needed therapy to prevent a thrombocytopenia).
2. other primary diseases causing thrombocytopenia and drug induced thrombocytopenia were excluded; if there were no abnormalities found in bone marrow smears, except a suspicion of an increased number of megakaryocytes. Drug induced thrombocytopenia was excluded by withholding drugs during clinical observation and careful anamnestic examination. Patients who were treated with heavy metal depot injections, which require treatment with chelating agents to see if this heavy metal is the cause of the thrombocytopenia, were excluded.
3. there was no splenomegaly.

This ITP group contained patients before and after splenectomy and with or without treatment with corticosteroids. (table 5). In some patients the marrow biopsy was carried out during a period of normal platelet counts following splenectomy.

Patients with hypoplastic thrombocytopenia (4)
Four patients with a diagnosis of hypoplastic thrombocytopenia had a history of a severe pancytopenia during which they needed blood transfusions. Marrow biopsy- and iron kinetic studies confirmed the diagnosis of bone marrow hypoplasia. Three of them improved markedly during treatment with androgens (oxymethalone). The other patient improved spontaneously. The marrow biopsy for this study was done at least one year after the start of the partial remission, in a period with normal or slightly decreased red cell production and leucocyte count but still with markedly reduced platelet count (table 6).

Patients with various kinds of thrombocytopenia (5)
Five patients with various kinds of thrombocytopenia have been studied. Their data are summarized in table 7. The platelet count in 3 patients was often less than at the date of biopsy.

Patients with thrombocytosis (4)
Thrombocytosis was defined as a platelet count of more than 310.000/µl (the mean platelet count of normal subjects, 208,000/µl, plus twice the standard deviation, 100,000/µl). The five patients with a thrombocytosis do not form a homogeneous group. There were cases with reactive or secondary thrombocytosis but also with primary or autonomous thrombocytosis (table 8).

Subjects studied for determination of megakaryocyte ploidy

Control subjects (12)
4 Normal volunteers and 8 patients with complaints or diseases in which no alterations of thrombocytopoiesis or megakaryocytopoiesis could be expected were examined (table 17).

Patients with chronic ITP (12)
12 Patients with (a history of) chronic ITP were examined. In six of them splenectomy had been performed. In 3 patients the platelet count was normal at the time of bone marrow sampling for ploidy determinations. In 2 of these

3 patients this was the result of therapy with corticosteroids (table 18). 2 Patients were examined before and after splenectomy; patient no. 19 some months after splenectomy; patient no.14 3 days after splenectomy.

Patients with chronic myeloid leukaemia (2)
The leukocyte counts of the patients with chronic myeloid leukaemia were 23,000 and 230,000/µl (table 19). One of them was treated with busulphan.

Patients with thrombocytopenia following cardiac surgery (3)
In three patients who developed a thrombocytopenia following cardiac surgery for prostheses of valves a marrow sample was taken the second or third postoperative day (table 19).

Patient with idiopathic thrombocytosis (1)
One patient with thrombocytosis and urticaria pigmentosa, who developed polycythaemia vera 2 years later, was examined (table 19).

2.2.2. Results

Cellularity of histological sections

In fig. 4 the cellularity of histological sections from the subjects is shown. The cellularity between subjects differed by a factor of 4 maximally. The differences in cellularity of bone marrow tissue can also be expected from the results in fig. 5 in which the part of a test field with fat vacuoles, sinusoids, vessels or ruptures of histological sections, indicated as "non-haemopoietic (cell) volume", is shown. This varied from 11.8% to 56.5%, mean 36.7% ± 10.2 (SD) of the test volume in 36 biopsies.

Total megakaryocyte volume uncorrected for cellularity

In 14 control subjects, the mean fractional volume of the test volume occupied by the collective volume of megakaryocytes was 0.71% ± 0.21 (table 4).

Lower values were obtained in patients with hypoplastic thrombocytopenia (fig.6). These values did not overlap the values obtained in patients with

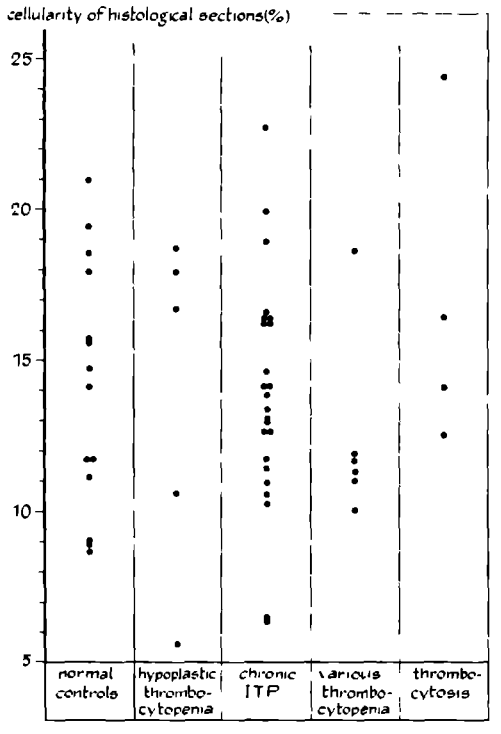

Fig. 4.Cellularity of marrow biopsies from normal controls and patients with (a history of) thrombocytopenia or thrombocytosis. The cellularity is expressed as the fractional volume of the bone marrow volume occupied by the collective volume of nuclei of haemotopoietic cells (excluding megakaryocytes).

(a history of) chronic ITP. In 24 marrow biopsies from 18 patients with (a history of) chronic ITP the mean total megakaryocyte volume uncorrected for cellularity was 1.34% ± 0.60 (SD) of the test volume (table 5). This is significantly increased in comparison with normal controls; p= 0.0002, using Wilcoxon's two-sample test.

The total megakaryocyte volume uncorrected for cellularity in the group of patients with various forms of thrombocytopenia was within the normal range (table 7, fig. 6).

In three of four patients with thrombocytosis the total megakaryocyte volume uncorrected for cellularity was three times greater than the mean normal value (table 8).

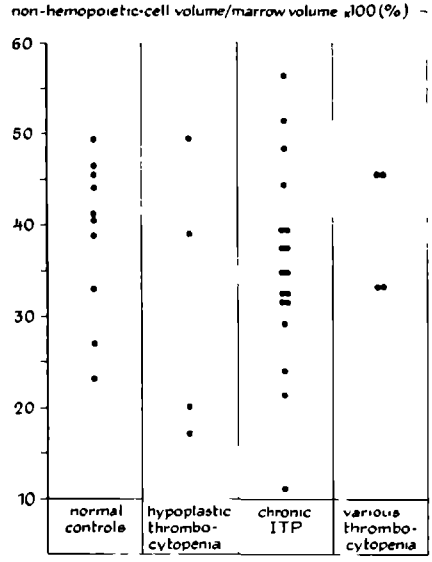

Fig.5.Non-haemopoietic (cell) volume in marrow biopsies from normal controls and patients with (a history of) thrombocytopenia or thrombocytosis. The non-haemopoietic volume is expressed as the fractional volume occupied by fat vacuoles, vessels, sinusoids and ruptures in histological sections.

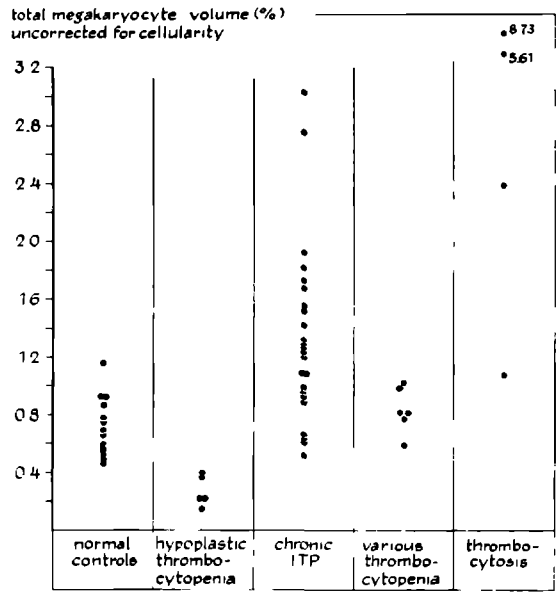

Fig.6.Total megakaryocyte volume in marrow biopsies uncorrected for cellularity in normal controls and patients with (a history of) thrombocytopenia or thrombocytosis. The total megakaryocyte volume is expressed as the fractional volume of the test volume occupied by the collective volume of megakaryocytes. In chronic ITP patients the total megakaryocyte volume is significantly increased in comparison with normal controls; p= 0.0002.

Table 4

Total megakaryocyte volume and number of sections in marrow biopsies from 14 normal controls.

Case no.	Sex	Age	Biopsy	Diagnosis	Platelet count* ($x10^3/\mu l$)	Megakaryocytes				
						total volume		number of sections		mean diameter of sections µm
						uncorrected for cellularity[1]	corrected for cellularity[2]	uncorrected for cellularity[3]	corrected for cellularity[4]	
48	m	33	24-05-72	Hypercalciemia	199	0.77	4.1	1.6	0.23	14.4
49	f	20	06-11-72	None	208	0.69	7.7	0.8	0.25	17.9
50	f	38	27-02-73	Osteoporosis?	173	0.47	5.2	0.9	0.27	16.1
51	m	52	06-06-72	Sprue	334	0.58	6.7	0.7	0.22	16.1
52	f	45	14-03-73	Hypercalciemia	189	0.87	6.1	1.1	0.21	17.0
53	m	25	03-04-73	Nephrolithiasis	216	0.93	5.9	1.2	0.22	18.2
54	f	40	19-03-73	Nephrolithiasis	287	1.16	5.6	1.3	0.17	17.3
55	f	41	26-01-73	Osteoporosis?	227	0.67	5.7	1.1	0.26	17.1
56	m	52	15-03-73	Osteoporosis	238	0.48	4.3	0.7	0.18	17.0
57	f	47	29-05-72	Nephrolithiasis	216	0.56	3.6	0.9	0.16	15.9
58	f	40	28-05-73	Nephrolithiasis	172	0.78	4.0	1.1	0.16	16.1
59	m	37	13-04-73	Hypercalciuria	125	0.93	6.0	1.0	0.19	18.1
60	m	47	09-04-73	Osteoporosis	316	0.54	3.0	0.7	0.10	18.1
61	m	29	21-09-73	Osteoporosis	132	0.52	4.6	0.6	0.14	16.3
Mean (n=14) ± SD					217	0.71 ±0.21	5.2±1.3	1.0 ±0.3	0.20±0.05	16.8±1.0

* Platelet count at the time of biopsy

For 1,2,3 and 4; see: expression of results of morphometrical determinations (2.2.1.)

Table 5

Total megakaryocyte volume and number of sections in marrow biopsies from 18 patients with ITP.

Case no.	Sex	Age	Biopsy	Splenectomy	Prednison (mg/day)	Platelet count* ($x10^3/\mu l$)	Megakaryocytes				
							total volume		number of sections		mean diameter of sections µm
							uncorrected for cellularity [1]	corrected for cellularity [2]	uncorrected for cellularity [3]	corrected for cellularity [4]	
1	m	41	06-07-72	-	-	16	1.67	10.2	1.8	0.31	16.8
			08-01-73	-	-	268	0.90	5.5	0.9	0.16	17.6
2	m	19	29-11-72	+	-	110	1.20	7.3	1.3	0.23	20.5
3	f	34	11-10-72	+	-	170	1.09	8.6	1.2	0.26	17.9
4	f	27	12-01-73	-	-	92	1.30	9.4	1.5	0.29	21.5
5	f	55	18-10-72	-	30	92	0.63	9.8	0.8	0.35	17.8
			25-01-73	+	-	156	1.28	9.6	1.3	0.26	22.6
6	m	59	07-09-72	-	60	58	0.92	9.0	1.2	0.34	18.3
			01-02-73	+	-	55	1.53	11.9	1.4	0.31	17.4
			20-09-73	+	-	66	1.23	9.7	1.6	0.35	22.9
7	f	65	04-05-72	-	25	56	0.61	5.6	0.9	0.22	19.7
			18-01-73	+	-	322	0.52	8.2	0.5	0.22	21.1
8	m	26	19-06-72	+	20	2	0.65	5.5	0.9	0.22	17.9
9	f	59	13-06-72	+	-	48	1.92	9.7	3.5	0.49	18.2
10	f	24	18-12-72	+	-	205	1.42	7.5	1.6	0.23	22.0
			12-06-73	+	-	200	1.75	6.8	1.8	0.22	20.8
11	f	26	20-02-73	+	-	166	1.81	11.1	2.4	0.41	20.3
12	f	45	28-08-72	+	10	23	2.75	16.8	3.0	0.51	20.9
13	f	65	22-06-72	+	5	8	1.25	8.5	1.2	0.23	20.4
14	m	30	11-01-73	-	-	10	3.03	21.5	3.5	0.68	21.4
15	f	33	04-07-73	+	30	10	0.98	11.1	1.1	0.29	18.6
16	f	49	21-05-73	+	10	10	1.23	9.1	1.5	0.33	20.5
17	f	57	15-10-73	-	-	7	1.55	11.6	2.3	0.43	19.8
18	f	22	04-10-73	-	-	35	1.09	8.9	1.7	0.44	20.3
Mean ±SD (n=24)						91±90	1.34±0.60	9.7 ±3.5	1.6±0.8	0.32±0.12	19.8±1.8
Normal range (mean± SD;n=14)						217	0.71±0.21	5.2 ±1.3	1.0±0.3	0.20±0.05	16.8±1.1

* platelet count at the time of biopsy

For 1,2,3 and 4; see: expression of results of morphometrical determinations (2.2.1.)

Table 6

Total megakaryocyte volume and number of sections in marrow biopsies from 4 patients with hypoplastic thrombocytopenia

Case no.	Sex	Age	Biopsy	Hb g/100 ml	Leucocyte count ($x10^3/\mu l$)	Platelet count * ($x10^3/\mu l$)	Megakaryocytes			
							total volume		number of sections	
							uncorrected for cellularity[1]	corrected for cellularity [2]	uncorrected for cellularity [3]	corrected for cellularity [4]
23	f	48	17-12-72	8.4	1.8	30	0.21	1.2	0.3	0.05
24	f	53	28-08-72	13.0	5.0	50	0.21	3.7	0.2	0.10
25	f	18	12-09-72	9.6	2.5	27	0.38	2.1	0.5	0.07
		19	19-09-73	8.2	2.9	34	0.39	2.2	0.5	0.07
26	f	19	18-09-72	13.0	4.5	19	0.15	1.4	0.2	0.06
Normal range (mean ± SD ; n = 14)						217	0.17± 0.21	5.2±1.3	1.0±0.3	0.20±0.05

*Platelet count at the time of biopsy

For 1,2,3 and 4; see: expression of results of morphometrical determinations (2.2.1.)

Table 7

Total megakaryocyte volume and number of sections in marrow biopsies from 5 patients with various thrombocytopenia

Case no.	Sex	Age	Biopsy	Diagnosis	Platelet count* ($x10^3/\mu l$)	Megakaryocytes			
						total volume		number of sections	
						uncorrected for cellularity[1]	corrected for cellularity[2]	uncorrected for cellularity[3]	corrected for cellularity[4]
27	f	23	19-06-72	Lupus erythematosus	15	1.02	8.6	1.3	0.30
28	f	62	23-10-72	Drug induced? (gold)	84	0.58	5.8	0.9	0.23
29	f	32	18-01-73	Thrombocytopathy	120 *	0.82	7.0	0.9	0.22
			08-03-73		113**	0.79	7.1	1.0	0.25
30	f	26	15-09-73	Familial thrombocytopenia	50	0.81	7.6	1.3	0.31
31	f	13	27-09-73	Familial thrombocytopenia	110**	0.96	5.5	1.3	0.20
Normal range (mean±SD ; n = 14)									

*Platelet count at the time of biopsy

** Platelet counts were often lower in other periods

For 1,2,3 and 4 ; see: expression of results of morphometrical determinations (2.2.1.)

Table 8

Total megakaryocyte volume and number of sections in marrow biopsies from 4 patients with thrombocytosis

Case no	Sex	Age	Biopsy	Diagnosis	Platelet count* ($x10^3$/ l)	Megakaryocytes			
						total volume		number of sections	
						uncorrected for cellularity[1]	corrected for cellularity [2]	uncorrected for cellularity[3]	corrected for cellularity [4]
32	f	46	10-05-73	Primary thrombocytosis	1,031	5.61	35.4	4.6	0.75
33	m	64	24-05-72	Urticaria pigmentosa	850	2.39	17.0	2.8	0.55
34	f	58	29-06-72	Rheumatoid arthritis	686	1.08	8.6	1.0	0.23
35	f	34	29-03-72	Osteomyelofibrosis	980	8.73	35.8	7.4	0.84
Normal range (mean+ SD; n = 14)					217	0.71±0.21	5.2+1.3	1.0±0.3	0.20±0.05

*platelet count at the time of biopsy

for 1,2,3 and 4; see: expression of results of morphometrical determinations (2.2.1.)

Table 9

Total megakaryocyte volume in mothers (with a history of) chronic ITP newborn with a thrombocytopenia

Mother					Newborn		
Case no.	Age	Course after splenectomy	Total megakaryocyte volume*	Platelet counts ($x10^3$/µl)	Platelet counts ($x10^3$/µl)	Haemorrhagic symptoms	Duration of thrombocytopenia
3	34	Platelet counts normal	8.6	170	6	Cephalhaematoma	2 months
10	25	No result of splenectomy; normalized after azathioprine	6.8	200	60	None	4 months
11	26	Mild thrombocytopenia	11.1	166	5	Petechiae some ecchymoses	4 months

*corrected for cellularity; normal range: 2.6-7.8

Total megakaryocyte volume corrected for cellularity

In 14 control subjects this value was 5.2% ± 1.3 (table 4). In 18 patients with chronic ITP (fig. 7) significantly greater values were obtained than in control subjects; p = 0.0001 (Wilcoxon's two sample test). The mean value in ITP patients

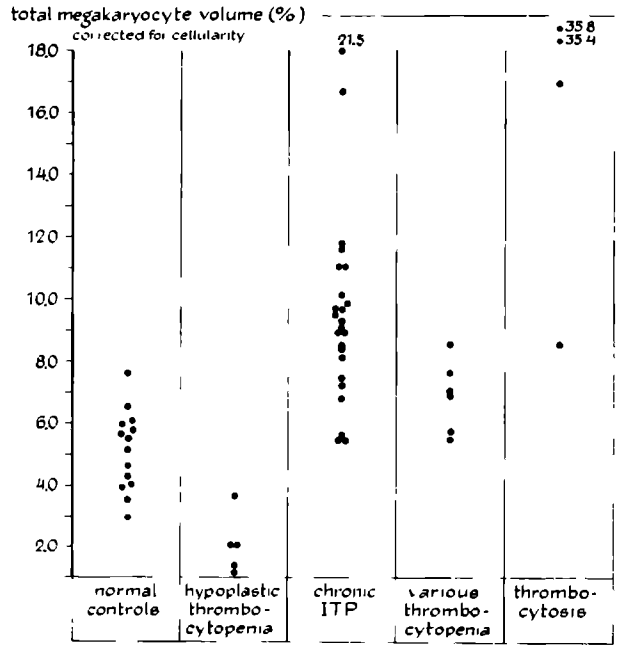

Fig. 7. Total megakaryoycte volume in marrow biopsies corrected for cellularity from normal controls and patients with (a history of) thrombocytopenia or thrombocytosis. The total megakaryocyte volume is expressed as the collective megakaryocyte volume/ collective volume of nuclei of hemopoietic cells (excluding megakaryocytes). In chronic ITP patients the total megakaryocyte volume is significantly increased in comparison with normal controls; p = 0.0001.

was 1.9 times greater than the mean value in normal controls. The results in patients with hypoplastic thrombocytopenia, thrombocytosis or various forms of thrombocytopenia were in accordance with the findings for total megakaryocyte volume uncorrected for cellularity (fig. 7, tables 4,5,6,7 and 8).

Total megakaryocyte volume during pregnancy in patients with a history of chronic ITP

Three patients with a history of chronic ITP were examined during pregnancy while there was a normal platelet count. The total megakaryocyte volume in 2 of the 3 patients was markedly increased, in the other patient a high normal

Table 10

Reproducibility of determinations of total megakaryocyte volume and number of sections of megakaryocytes in marrow biopsies. Two marrow biopsies were taken at different times from each of 4 subjects

Case no.	Biopsy	Total megakaryocyte volume		No. of sections of megakaryocytes	
		uncorrected for cellularity[1]	corrected for cellularity [2]	uncorrected for cellularity [3]	corrected for cellularity [4]
29	18-01-73	0.82	7.0	0.9	0.22
	08-03-73	0.79	7.1	1.0	0.25
10	18-12-72	1.42	7.5	1.6	0.23
	12-06-73	1.75	6.8	1.8	0.22
6	01-02-73	1.53	11.9	1.4	0.31
	20-09-73	1.23	9.7	1.6	0.35
25	12-09-72	0.38	2.1	0.5	0.07
	19-09-73	0.39	2.2	0.5	0.07

1,2,3 and 4: see expression of results of morphometrical determinations

[1]normal value 0.71 ± 0.21

[2]normal value 5.2 ± 1.3

[3]normal value 1.0 ± 0.3

[4]normal value 0.20 ± 0.05

Table 11

Influence of megakaryocyte size on the relationship true diameter: diameter of cross-sections of megakaryocytes in a serial section study of 101 megakaryocytes of an ITP patient (no.9)

Size classes range of true diameter (µm)	number of megakaryocytes	mean true diameter	mean section diameter	true diameter / section diameter
12 - 17	5	14.5	12.9	1.13
17 - 20	25	18.3	15.2	1.20
20 - 23	26	21.3	17.0	1.25
23 - 26	30	24.0	18.9	1.27
26 - 30	12	27.5	21.3	1.29
30 - 39	3	34.9	25.0	1.39
mean (n=101)		22.2	18.3	1.21

value was found. The offsprings of all these 3 patients suffered from a neonatal thrombocytopenia (table 9).

Total megakaryocyte volume in relation to age, splenectomy and platelet count
The age of the subjects examined varied between 13 and 65 years. There was no relationship between the total megakaryocyte volume and the age in normal controls and chronic ITP patients (table 4 and 5).

In chronic ITP patients there was no relationship between the total megakaryocyte volume and platelet count (table 5).

In chronic ITP patients the estimation of the total megakaryocyte volume was performed 8 times before splenectomy and 16 times after splenectomy. There was no difference found in the total megakaryocyte volume before and after splenectomy (table 5).

Reproducibility of total megakaryocyte volume estimations
Two biopsies from each of 4 patients obtained with a minimal time interval of two months, were available. One of them (no. 10) was pregnant when the second biopsy was carried out. One of them (no. 6) was not in remission after splenectomy. The platelet counts of these patients were rather constant. The two other patients also seemed to be in a steady state. The total megakaryocyte volume was very constant in 3 of 4 patients (table 10).
For practical reasons there was some difference in duration of dehydration time in 70% ethanol. The influence on the estimation of the total megakaryocyte volume was studied by dividing a biopsy in two parts, one part was dehydrated for the normal time of 1 hour in 70% ethanol and the other for 2 days. The difference between the total megakaryocyte volume estimations in the two parts of the biopsy was 6%.

Megakaryocyte size

a. True diameter
In a serial section study of 101 megakaryocytes from an ITP patient, 460 sections were recognized as parts of megakaryocytes on photomicrographic enlargements. The true diameters of these 101 megakaryocytes varied from 12.4 to 39.1µm. The mean true diameter was 22.2 µm ± 4.1 µm (SD) (table 11).

b. Mean section diameter

b.1 Measurements on photomicrographs

In the above mentioned serial section studies (photomicrographs) the diameter of the smallest sections was 7.2 µm. The mean diameter of all sections from 101 serially sectioned megakaryocytes was 18.3 µm (table 11).

Some at random chosen photomicrographs of the same serial section study were examined. The mean section diameter of 97 sections of megakaryocytes from these microphotographs was 20.6 µm (table 12).

Table 12

Comparison of diameters (µm) in serial and random section studies measured on photomicrographs and measured with an ocular micrometer in 101 megakaryocytes and their sections from ITP patient (no.9)

Type of study	Photomicrographs		Ocular micrometer	True diameter / section diameter
	mean true diameter	mean section diameter	mean section diameter	
Serial sections	22.2	18.3		1.21
Random sections		20.6		1 08
			18.2	1.22

Table 13

Comparison of diameters of random sections of megakaryocytes measured on photomicrographs and measured with an ocular micrometer in 2 control subjects and a patient with chronic ITP

Case no.	Mean diameter of sections		
	photomicro-graphs µm	ocular micrometer µm	photo / oc. meter
9	20.6	18.2	1.13
55	20.1	17.1	1.17
56	20.8	17.0	1.22

Results of measurements of diameters of more than 100 sections of megakaryocytes on microphotographs from two other subjects are given in table 13.

Ratio true diameter mean section diameter

The factual influence of the true diameter of a megakaryocyte on the quotient

$\frac{\text{true diameter}}{\text{mean section diameter}}$ is given in table 11

The quotient varied from 1.13 for the smallest megakaryocytes to 1.39 for the largest megakaryocytes in serial sections. Decrease in the size of megakaryocytes resulted in a decrease in the difference between true diameter and mean diameter of sections in serial section studies (fig. 8). The quotient

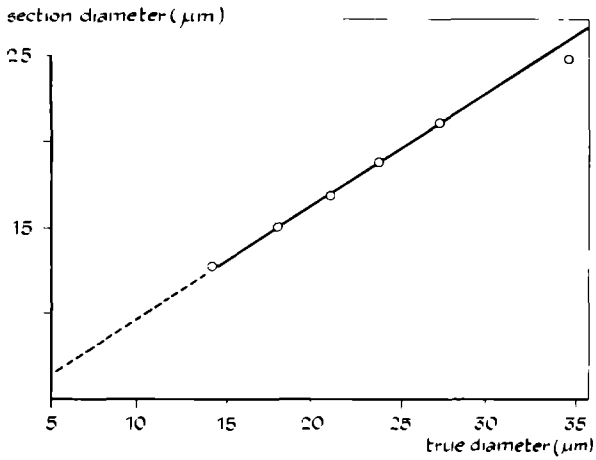

Fig.8. Relationship between the true diameter of megakaryocytes and the mean diameter of sections of megakaryocytes in a serial section study of 101 megakaryocytes from a patient with chronic ITP (no.9), categorized in 6 size classes of true diameters

$\frac{\text{true diameter}}{\text{mean section diameter}}$ was $1.08\left(\frac{22.2}{20.6}\right)$ if the sections of megakaryocytes were measured on at random duplicated photographs of the serial section study (table 12).

As described above (2.1.2.) the theoretical relationship between true diameters of spheres and diameters of sections is:

$$\bar{r} = \frac{\pi}{4\bar{m}} \qquad (2)$$

Application of this formula to the data obtained from measurements on 460 serial sections resulted in a mean true diameter of 28.2 μm. The calculated mean true diameter from the 97 random sections of the serial section study resulted in a mean true diameter of 35.4 μm.

b.2. Measurements of diameters of sections of megakaryocytes by ocular micrometry.

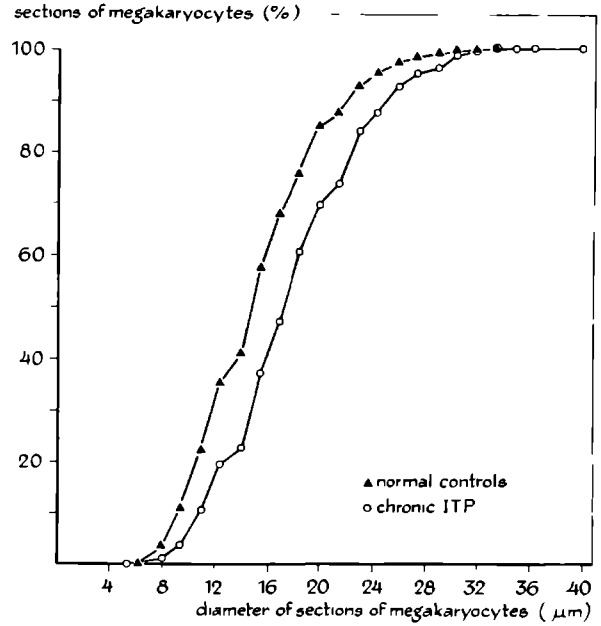

Fig.9.Cumulative diameter distribution curve of a total of 3594 sections of megakaryocytes in marrow biopsies, measured with an ocular micrometer, in 14 normal controls and in 18 patients with (a history of) chronic ITP

The mean section diameter of approximately 100 - 125 random sections of megakaryocytes measured in each subject varied in 14 normal controls between 14.4 and 18.2 µm, and in 18 chronic ITP patients from 16.8 to 22.9 µm (fig.10). This difference was significant, p= 0.0001, using Wilcoxon's two sample test (fig. 10). The mean diameter of the individual values in the group of normal controls was 16.8 µm ± 1.1 (SD), and in ITP patients 19.8 µm ± 1.8 (SD) (table 4 and 5). The section diameters varied between 6 and 40 µm, with a top level between diameters of 16 and 21 µm. Sections with diameters less than 10 µm were rarely recognized as parts of megakaryocytes.

The reproducibility of the estimation of the mean diameter of sections of megakaryocytes from a single biopsy, measured with an ocular micrometer, is given in table 14.

The influence of a different dehydration time, which occured in practice, was studied by dividing a biopsy in two parts, one part was dehydrated for the normal time of 1 hour, the other part was kept for 2 days in 70% ethanol.

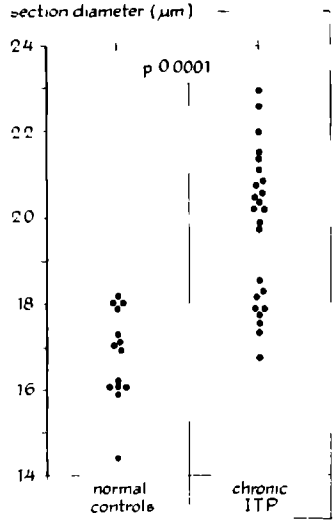

Fig.10 Mean diameters of sections of megakaryocytes measured with an ocular micrometer in 14 normal controls and in 18 patients with (a history of) chronic ITP

Table 14

Reproducibility of measurements of mean diameter (μm) using an ocular micrometer on the same histological sections from a single biopsy in 4 subjects by one investigator

Case no.	Result of various measurements			Mean
9	17.3	17.9	19.4	18.2
10	21.6	22.4		22.0
58	15.5	16.0	16.8	16.1
59	17.3	18.9		18.1

In the two parts of the biopsy the mean diameter of 125 sections of megakaryocytes was respectively 17.0 μm and 16.9 μm.

Comparison of mean section diameter obtained from measurements on photomicrographs and measurements by ocular micrometry was carried out in 3 biopsies. As is shown in table 13 the mean diameter of about 100 sections of megakaryocytes measured on photographs was 1.13 to 1.27 times greater than the diameter measured by studying histological sections more directly by ocular micrometry.

c. Mean area of sections of megakaryocytes

This parameter for megakaryocyte size was significantly greater in patients with (a history of) chronic ITP in comparison with normal controls (p=0.04) (fig.11).

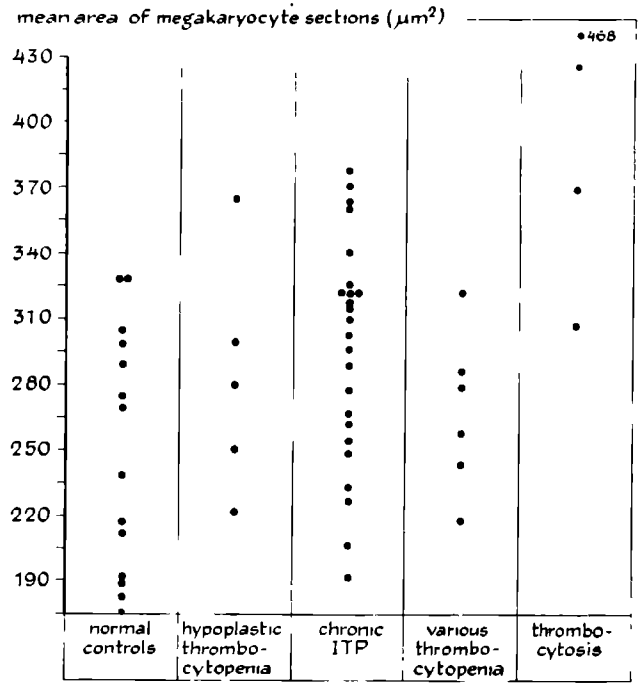

Fig.11 Mean area of sections of megakaryocytes in marrow biopsies from normal controls and patients with (a history of) thrombocytopenia or thrombocytosis. In chronic ITP patients the mean area of sections of megakaryocytes is significantly increased in comparison with normal controls; p= 0.04

Megakaryocyte size in relation to platelet count, splenectomy, total megakaryocyte volume and megakaryocyte count

With an increasing mean diameter of the megakaryocyte sections there was some increase in the total megakaryocyte volume. However there was no significant correlation between the different functions of megakaryocytic size and the blood platelet count or the total megakaryocyte volume in ITP patients. No difference was found in the functions of megakaryocytic size in ITP patients before and after splenectomy (table 5).

Number of sections of megakaryocytes in histological sections

The number of sections of megakaryocytes uncorrected for cellularity in

14 normal controls varied between 0.6 and 1.6 per test area with a mean value of 1.0 ± 0.3 (SD) (table 4).

In patients with a history of aplastic anaemia lower figures were obtained (table 6).

The number of sections of megakaryocytes uncorrected for cellularity in patients with chronic ITP showed a mean value of 1.6 ± 0.8 (SD). This differed significantly from normal controls, p= 0.001, using Wilcoxon's two sample test (table 5).

In patients with various other forms of thrombocytopenia the number of sections of megakaryocytes was in the normal range (table 7). The greatest number of sections of megakaryocytes was found in patients with a thrombocytosis (table 8).

Number of sections corrected for cellularity

Number of sections corrected for cellularity from the examined groups of subjects is given in fig. 12. There was a significant difference between normal

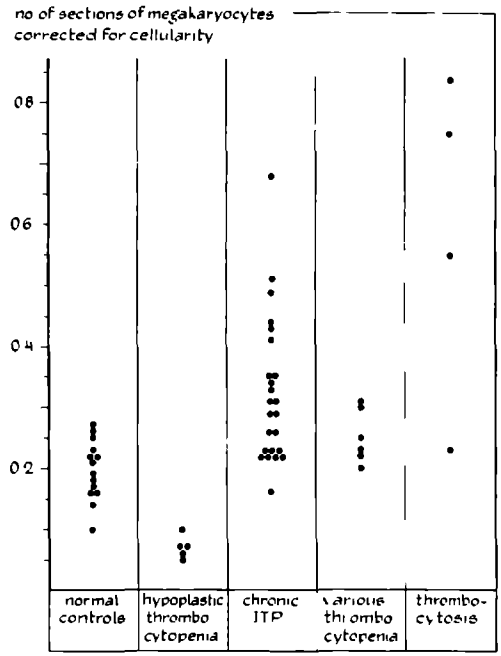

Fig.12. Number of sections of megakaryocytes, corrected for cellularity, in marrow biopsies from normal controls and patients with (a history of) thrombocytopenia or thrombocytosis. In chronic ITP patients the number of sections of megakaryocytes is significantly greater than in normal controls; p= 0.0003

controls and ITP patients (p=0.0003). In normal controls the mean value of the number of sections of megakaryocytes corrected for cellularity was 0.20 ± 0.05 (SD), in chronic ITP patients 0.32 ± 0.12 (SD).

Reproducibility of megakaryocyte count

In each of 4 patients there were 2 biopsies available which were taken with some time interval, while they seemed to be in a clinically steady state. The counting results are rather constant as is shown in table 10.

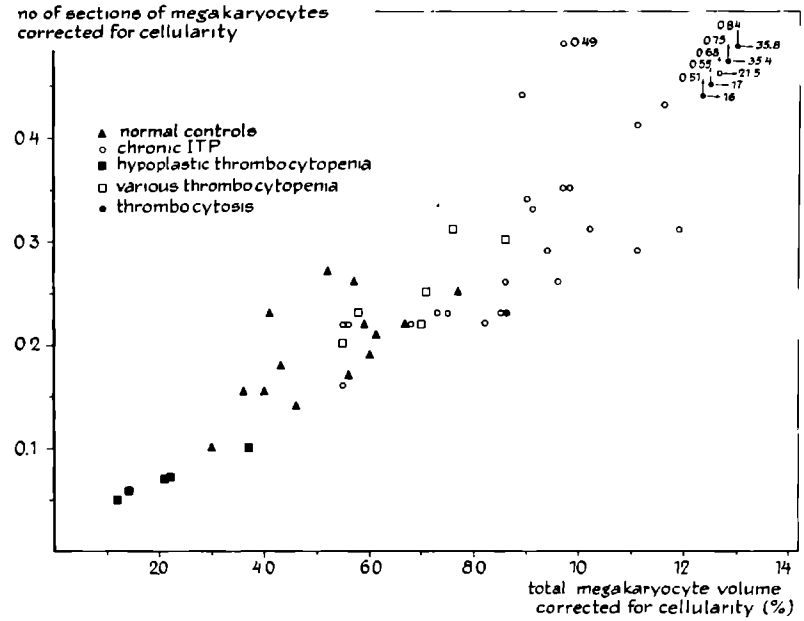

Fig.13. Relationship between megakaryocyte count and total megakaryocyte volume in marrow biopsies from normal controls and patients with (a history of) thrombocytopenia or thrombocytosis. The regression line is defined by y=0.0225 x+0.0859. The correlation coëfficient is 0.928

Megakaryocyte count in relation to other morphometrical parameters and clinical data

There was a good positive relationship between the number of megakaryocytes corrected for cellularity and the total megakaryocyte volume in marrow biopsies of normal controls and various patients (fig. 13).

With an increase in diameter of the sections of megakaryocytes there was some increase in the number of sections of megakaryocytes (fig. 14). However this correlation was not significant. The number of sections of megakaryocytes before and after splenectomy was the same. There was no relationship found between the number of sections and the platelet count or subject's age.

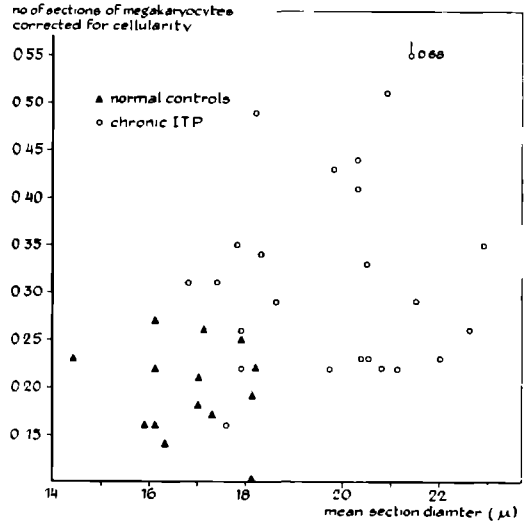

Fig.14. Relationship between mean diameter of sections of megakaryocytes and the megakaryocyte count in normal controls and in patients with (a history of) chronic ITP. The regression line is defined by 0.0235 x - 0.1630. The correlation coëfficient is 0.425

Megakaryocyte ploidy

The reproducibility of the determination of the distribution of megakaryocytes in ploidy classes and mean ploidy values from six marrow aspirates is given in table 15. In table 16 the reproducibility of ploidy determinations from two control subjects is shown; in each subject two marrow aspirations were taken at different times.

Control subjects

In 9 out of 12 control subjects a frequency peak was visible in the 16 N class. The mean ploidy values varied between 19.6 N and 29.6 N. The percentages of megakaryocytes in various ploidy classes and the mean ploidy values of megakaryocytes from each subject is given in table 17. A histogram of ploidy values of megakaryocytes in a normal subject is given in fig. 15.

Patients with chronic ITP

In comparison with control subjects the percentages of megakaryocytes in various ploidy classes in 12 patients with (a history of) ITP showed a shift from the 16N class toward the 32N class (table 18, fig.16). The mean ploidy values of each subject varied between 19.3N and 31.2N. These ploidy values were increased in comparison with control subjects (fig. 17). Using Wilcoxon's two sample test

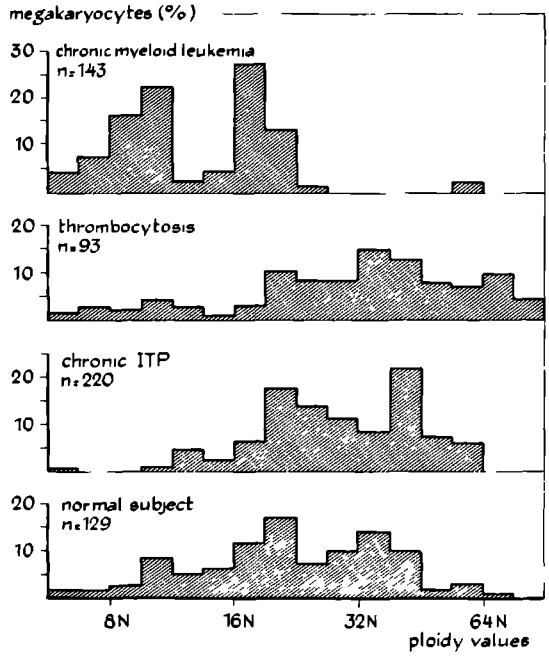

Fig.15.Histograms of ploidy values, determined by microfluorometry of Feulgen stained megakaryocytes, in a normal subject, a patient with chronic ITP, a patient with primary thrombocytosis and a patient with chronic myeloid leukaemia. The ploidy value was calculated using metamyelocytes as 2N reference. Ploidy values are plotted on a logarithmic scale. N: The number of measured megakaryocytes

p was 0.057. The average of the mean ploidy values in the ITP group was 26.5N, in the control subjects 23.9N (table 18). A histogram of ploidy values in an ITP patient is given in fig. 15.

Patients with chronic myeloid leukaemia

The 2 patients suffering from chronic myeloid leukaemia showed a shift toward lower ploidy values (table 19). The mean ploidy of each subject did not overlap the values obtained in control subjects (fig. 17). In one of these two patients a mean ploidy value of 13N was measured. This was the lowest value observed in this study.

Patients with thrombocytopenia following cardiac surgery

In 3 patients with acute thrombocytopenia some days after cardiac surgery, normal ploidy values of the megakaryocytes were obtained (table 19).

Table 15

Reproducibility of microfluorometric determinations of ploidy values of Feulgen-stained megakaryocytes from a single marrow aspirate in 6 subjects

Case no.	Megakaryocytes (%)				Mean ploidy (N)
	8N	16N	32N	64N	
1	3.1	36.7	54.7	5.5	27.1
	5.1	45.8	35.6	13.6	27.8
5	9.6	26.1	48.7	15.7	30.6
	17.6	26.4	49.5	6.6	25.7
20	2.8	33.3	47.2	16.7	31.3
	-	28.6	61.6	9.8	30.6
37	18.2	58.6	22.2	1.0	18.6
	27.0	49.5	20.7	2.7	18.4
62	20.0	27.3	44.5	8.2	25.5
	20.2	34.6	38.5	6.7	23.8
72	21.8	38.6	30.7	8.9	23.4
	26.0	42.7	24.0	7.3	21.3

Table 16

Reproducibility of microfluorometric determinations of ploidy values of Feulgen-stained megakaryocytes in 2 subjects. Marrow aspirates were not taken simultaneously

Case no.	Marrow aspirate	Megakaryocytes				Mean ploidy (N)
		8N	16N	32N	64N	
62	10-03-72	14.0	39.5	41.1	5.4	24.0
	04-07-73	20.1	32.2	40.7	7.0	24.3
65	10-03-72	14.8	30.4	39.1	15.7	28.6
	10-05-72	8.6	44.8	33.3	13.3	27.0

Table 17

Percentage of megakaryocytes in various ploidy classes and mean ploidy of megakaryocytes from each subject in 12 control subjects

Case no.	Sex	Age	Marrow aspirate	Diagnosis	Megakaryocytes %				Mean ploidy (N)
					8N	16N	32N	64N	
62	m	36	10-03-72	-	14.0	39.5	41.1	5.4	24.0
			04-07-73	-	20.1	32.2	40.7	7.0	24.3
63	f	40	28-05-73	Nephrolithiasis	8.8	61.9	27.4	1.8	20.5
64	f	25	10-07-73	Hypertension	19.3	42.2	33.9	4.6	22.1
65	m	28	10-03-72	-	14.8	30.4	39.1	15.7	28.6
			10-05-72	-	8.6	44.8	33.3	13.3	27.0
66	f	34	26-05-73	Diarrhea	5.8	23.3	62.1	8.7	29.6
67	m	29	16-10-72	-	14.9	40.6	37.6	6.9	22.1
68	m	37	09-10-73	Nephrolithiasis	17.9	53.6	28.6	-	19.2
69	m	36	16-05-72	Gastritis?	22.5	39.2	39.2	1.0	21.3
70	f	16	13-06-72	Anorexia nervosa	19.4	51.6	28.3	0.8	19.3
71	m	46	12-07-73	Hepatitis	23.4	39.6	27.9	9.0	22.9
72	m	34	10-03-72	-	12.4	40.2	37.1	10.3	25.9
73	f	58	24-04-72	-	10.3	33.3	44.4	12.0	28.0
Mean (n=14)					15.1	40.8	37.2	6.9	23.9
SD					5.5	9.9	9.0	4.7	3.4

Table 18

Percentage of megakaryocytes in various ploidy classes and mean ploidy of megakaryocytes from each subject in 12 patients with (a history of) chronic ITP

Case No.	Sex	Age	Marrow aspirate	Splenectomy	Prednison mg/day	Platelet count ($x10^3/\mu l$)	Megakaryocytes %				Mean ploidy (N)
							8N	16N	32N	64N	
1	m	41	06-07-72	-	-	16	4.5	40.4	46.5	8.5	27.5
			04-06-73	+	-	213	9.7	49.6	35.4	5.3	23.4
5	f	55	28-07-72	-	-	38	13.2	27.3	47.3	12.3	28.4
6	m	59	04-09-72	-	60	29	9.4	47.8	39.1	3.6	23.2
7	f	65	04-05-72	-	25	56	7.7	36.5	51.0	4.8	25.8
8	m	26	19-06-72	+	20	2	3.6	33.9	47.2	15.2	30.5
11	f	26	20-02-73	+	-	166	4.5	24.2	58.4	12.9	31.2
12	f	45	17-04-72	+	5	16	7.3	41.7	48.9	2.1	24.6
13	f	65	13-03-72	+	5	14	4.2	42.7	50.1	3.1	25.8
14	m	30	11-01-73	-	-	10	6.9	33.7	55.4	4.0	26.2
			03-03-73	+	80	188	4.8	33.7	56.7	4.8	27.0
18	f	22	04-10-73	-	-	35	29.0	38.0	32.0	1.0	19.3
19	m	13	28-09-72	-	-	20	7.1	34.8	50.0	8.0	27.3
20	f	20	05-06-72	-	15	80	1.4	30.9	54.5	13.2	30.9
Mean (n= 14)							8.1	36.7	48.3	7.1	26.5
SD							6.7	7.2	7.9	4.6	3.3
Normal range: mean (n=14)						(208)	15.1	40.8	37.2	6.9	23.9
		SD				(50)	5.5	9.9	9.0	4.7	3.4

Table 19

Percentage of megakaryocytes in various ploidy classes and mean ploidy of megakaryocytes from each subject in patients with chronic myeloid leukaemia (CML), thrombocytopenia following cardiac surgery or thrombocytosis

Case no.	Sex	Age	Marrow aspirate	Diagnosis	Platelet count ($x10^3/\mu l$)	Megakaryocytes %				Mean ploidy (N)
						8N	16N	32N	64N	
33	m	64	24-05-72	thrombo-cytosis	850	10.5	16.8	44.1	28.7	36.0
36	m	53	23-11-72	CML*	673	50.5	46.2	1.0	2.0	13.0
37	f	61	12-07-73	CML*	295	23.3	52.9	21.4	2.4	18.5
38	m	43	02-03-73	ASD**	83	17.3	57.2	24.9	1.0	19.1
39	m	49	29-03-73	AI ***	80	13.8	27.0	50.0	9.2	27.3
40	m	30	29-03-73	AI	64	20.0	42.1	27.4	10.5	23.8
Normal range: mean (n=14)					208	15.1	40.8	37.2	6.9	23.9
SD					50	5.5	9.9	9.0	4.7	3.4

*CML: chronic myeloid leukaemia, **ASD: atrial septal defect, *** aortic insufficiency

Patient with thrombocytosis

In a patient with primary thrombocytosis and urticaria pigmentosa an increased number of 64 N megakaryocytes was observed with an increased mean ploidy value of 36.0 N (table 19, fig. 17).

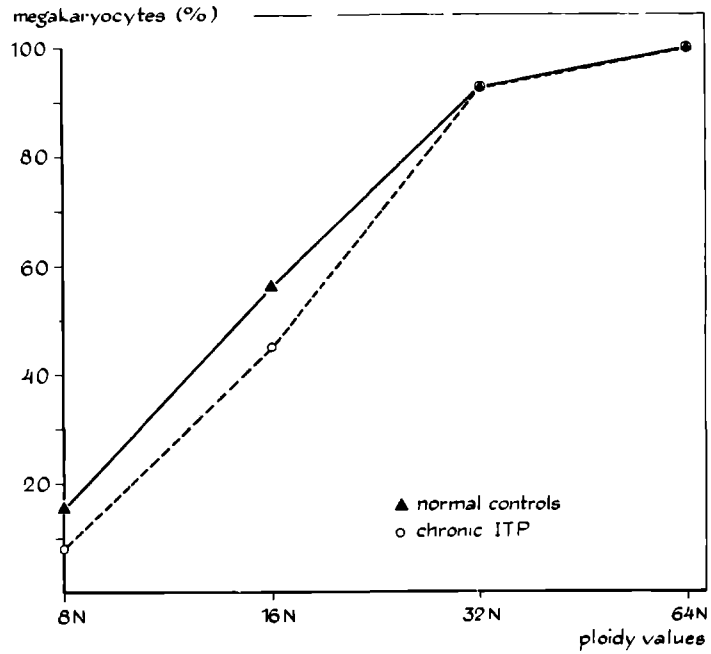

Fig.16.Cumulative ploidy distribution of megakaryocytes in normal controls and in patients with (a history of) chronic ITP. Ploidy is determined by microfluorometry of Feulgen stained nuclei.

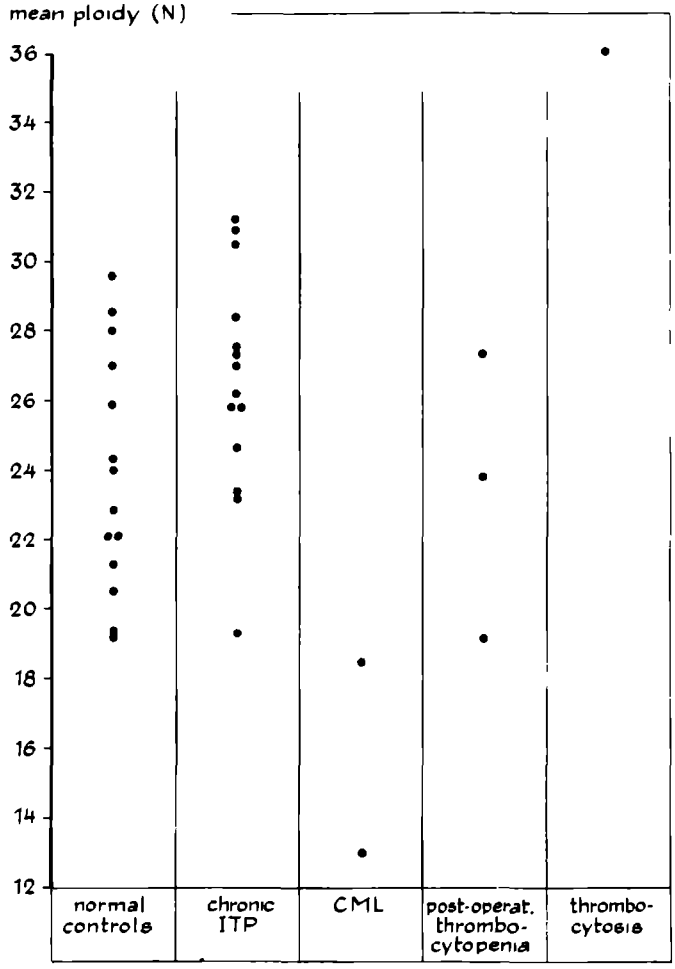

Fig.17 Mean ploidy values of megakaryocytes from each subject, determined by microfluorometry of Feulgen stained nuclei, in normal controls and in patients with (a history of) chronic ITP, chronic myeloid leukemia, thrombocytopenia following cardiac surgery or thrombocytosis

2.2.3. Discussion

Morphometrical aspects

In patients with chronic ITP an increase in total megakaryocyte volume (1.9x), megakaryocyte size (mean section diameter 1.17 x) and number of sections of megakaryocyte (1.6x) has been observed. However some errors occur in morphometry:

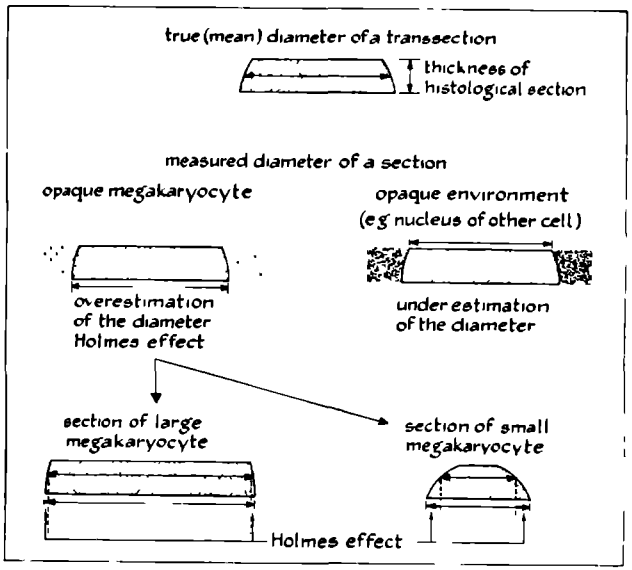

Fig.18. Illustration of the effect of environmental opacity on the determination of megakaryocytic section diameters in histological preparations

1. Not recognizing small sections of megakaryocytes

When the diameter of transsections of megakaryocytes is less than about 10 µm these pieces of the megakaryocytes are not recognized (Penington et al. 1970; Harker 1968a). Also in our study only a few sections of megakaryocytes with a diameter less than 10 µm were found (fig. 9).

2. Holmes effect

Tissue slices are investigated by transmitted light and measurements are done on projected images. The projection area of opaque objects will occupy a larger fraction of the field of observation than corresponds to their volumetric contribution (fig. 18). This overestimation was first described by Holmes (1927). The degree of this overestimation depends on the diameter of the megakaryocytes (fig. 18) and the thickness of the section (Weibel 1967).

3. Overlap of opaque structures

When there is an overlap of a part of a megakaryocyte by an opaque structure in the histological section, for instance nucleated cells, this section of the megakaryocyte will not be recognized or such an overlap can result in a decrease in the recognized area of the megakaryocyte (fig. 18).

Therefore the influence of these errors on the results of the determinations of megakaryocyte size (a), the total megakaryocyte volume (b) and the number of sections of megakaryocytes (c) in marrow sections will be discussed.

a) Significance of the observed differences in megakaryocyte size between ITP patients and control subjects

In ITP patients the mean section diameter and mean area of sections of megakaryocytes were greater than in control subjects. However, in theory determinations of these parameters for megakaryocyte size may be influenced by the 3 above mentioned errors. The Holmes effect (2) and not recognizing small sections of megakaryocytes (1) cause an overestimation of the megakaryocyte size, whereas the overlap of opaque structures (3) causes an underestimation.

The existence of errors 1 and 2: could be demonstrated in the following manner:

Error 1) In serial section studies (with the aid of the photographs of adjacent sections of the same megakaryocytes) more sections were recognized than in random section studies of the same photographs.

Error 2) The total volume of megakaryocytes from the serial section study calculated with the largest section diameter of each individual megakaryocyte, assuming a spherical form of the megakaryocyte, was about equal to the total volume of the recognized sections of these megakaryocytes calculated with the diameter of each section, assuming a cylindrical form of the section of the megakaryocyte. However the total volume calculated from all recognized sections should be much less than the total volume calculated from the sections with the largest diameter of each megakaryocyte, because not all sections are recognized. Therefore, the measured diameters of sections of megakaryocytes are overestimated in comparison with the measured maximal diameter of megakaryocytes, which is explained by the Holmes effect.

The mean true diameter calculated from the diameters of sections employing the formula (2) $\bar{r} = \frac{\pi}{4\bar{m}}$ (section 2.1.2.) was greater than the measured mean

true diameter. This is also in accordance with an overestimation of the measured section diameter due to the Holmes effect and to not recognizing small sections.

The influence of error 3 is difficult to determine. It will be more important in small megakaryocytes than in large megakaryocytes.

So in small megakaryocytes error 1 and 2 are more important while error 3 is less important (fig. 18). Therefore, comparing sections of large and small megakaryocytes, the effect of the 3 errors will be cumulative and will diminish the difference in the mean section diameter.
From the foregoing discussion it may be concluded that the differences in size between megakaryocytes of ITP patients and of normal subjects are greater than the observed differences in mean section diameters of megakaryocyte suggest.
The observed increase in megakaryocyte size in ITP patients agrees with the results of Harker et al. (1969) and Albrecht et al. (1974).
The differences in diameter a) between those measured and calculated (formula 2), b) from serial and random sections, c) from microphotography and ocular micrometry, can be explained in terms of the 3 mentioned errors.
The importance of these errors will be influenced by the recognizibility of the sections which depends on factors such as the focusing of the microscopical image and the colour of the microscopical image in contrast to the photographic image.

b) Influence of diameter of megakaryocytes on morphometrical determination of total megakaryocyte volume
An increase in diameter of megakaryocytes results in an increase in the percentage of sections of megakaryocytes which are recognized. Therefore it is questionable whether the increase in total megakaryocyte volume of ITP patients, found in this study, is reliable. To resolve this question we calculated the influence of the diameter of megakaryocytes on the results of the determination of the total volume of megakaryocytes for some arbitrarily chosen size classes of megakaryocytes (table 20).
The megakaryocyte diameters 15,20, 25 and 30 µm were chosen because in our material the measured diameter in serial and random section studies were in this range and were in accordance with the range found by Harker et al. (1969).
The theoretical number (N) of sections of a megakaryocyte with a radius R in histological sections of 5 µm thickness (T), can be calculated with the formula developed in section 2.1.1.

$$N = \frac{2R}{T} + 1 \qquad (1)$$

From this theoretical number of sections only sections with a certain minimal size will be recognized. The radius of the smallest recognized section is indicated by r. The percentage of the theoretical number of sections which will be recognized can be calculated with the formula: (Weibel, 1967):

$$\frac{100}{R}\sqrt{R^2 - r^2} \qquad (11)$$

For megakaryocytes with diameters of 15, 20, 25 and 30 µm the theoretical number of sections is respectively 4,5,6 and 7. If only sections with a diameter of > 10 µm will be recognized the percentage of sections recognized: the

Table 20

Theoretical influence of the number of sections, the real and measured volumes and Holmes effect in serially sectioned (5 µm thickness) megakaryocytes of different size classes, assuming that sections of megakaryocytes with a diameter of less than 10 µm are not recognized

	True diameter of megakaryocytes (µm)			
	15	20	25	30
number of sections per megakaryocyte				
theoretical number	4	5	6	7
% of theoretical number with a diameter >10 µm	75%	86%	92%	94%
true volume of megakaryocytes				
whole megakaryocyte (μm^3)	1767	4189	8181	14137
recognized part (section diameter >10 µm), as % of true volume	91%	97%	99%	100%
Holmes effect-volume				
of whole megakaryocyte, as % of whole, true volume	50%	38%	30%	25%
of recognized part (section diameter >10 µm), as % of recognized true volume	15,4%	19,9%	19,4%	18,0%
Measured volume, as % of true volume (recognized part + Holmes effect of recognized part)	105%	116%	119%	118%

theoretical number of sections of megakaryocytes, is respectively 75%, 86%, 92% and 94% (table 20). This is in good agreement with the percentage of recognized sections found in the serial study of 101 megakaryocytes (84%).

The true volume of megakaryocytes of the mentioned 4 megakaryocyte size classes in table 20 was calculated with the formula:

$$0.5236\ (2R)^3 \qquad (3)$$

The volume of the recognized part of megakaryocytes (section radius >r) was calculated with the formula:

$$2.\int_r^R \pi\ x^2\ d\left(\sqrt{R^2 - x^2}\right) = \frac{2}{3}\ \pi\ (2R^2 + r^2)\sqrt{R^2 - r^2} \qquad (12)$$

where $r < x < r$ is.

The calculation of the overestimation of the recognized part due to the Holmes effect is based on the following considerations:

If spheres with a radius R are sectioned, circular profiles with a radius x are obtained at a distance of

$$\sqrt{R^2 - x^2}$$

from the centre. Such profiles occur with a probability of

$$\frac{x}{R\sqrt{R^2 - x^2}}$$ (Weibel, 1967)

The volume of a cross-section with thickness T was estimated as $\pi\ x^2\ T$, where x is the maximum radius of the cross-section.

All cross-sections containing the centre of the sphere have the maximum radius R. Their probability of occurring is $\frac{T}{2R}$. Their volume was estimated as $\pi\ R^2\ T$. The estimated mean volume of cross-sections with a maximum radius >r, can therefore be calculated as:

$$\int_r^R \frac{x}{R\sqrt{R^2 - x^2}} \cdot \pi\ x^2\ T\ dx + \frac{T}{2r} \cdot \pi\ R^2\ T =$$

$$\frac{1}{3}\ \pi\ \frac{T}{R}\ (2\ R^2 + r^2)\sqrt{R^2 - r^2} + \frac{1}{2}\ \pi\ R\ T^2$$

To obtain the estimated volume of the sphere this mean cross-section volume was multiplied by the mean number of cross-sections from the recognized part of the sphere, being

$$\frac{2\sqrt{R^2 - r^2}}{T}$$

resulting in the following formula for the estimated volume of the sphere:

$$\frac{2\pi}{3R}\left(2R^2 + r^2\right)\left(R^2 - r^2\right) + \pi R T\sqrt{R^2-r^2}$$

Without the Holmes-effect the recognized volume of the sphere is (see 12):

$$\frac{2}{3}\pi\left(2R^2 + r^2\right)\sqrt{R^2-r^2}$$

The overestimation of the recognized part due to the Holmes effect can therefore be calculated with the formula:

$$\frac{2\pi}{3R}\left(2R + r^2\right)\left(R^2 - r^2\right) + \pi R T\sqrt{R^2 - r^2} - \frac{2}{3}\pi\left(2R^2 + r^2\right)\sqrt{R^2-r^2} \quad (13)$$

The result of the calculations show that in theory the determination of the total megakaryocyte volume with the employed morphometrical method, estimating the surface of transsections of megakaryocytes, results in an overestimation of the total megakaryocyte volume by a factor of between 5% and 19% for megakaryocytes with diameters between 15 and 30 µm (table 20).
However the difference in diameters between megakaryocytes of ITP patients and control subjects will be much less than 15 µm because:

a. The difference in true diameters between the smallest and the largest megakaryocytes proved to be only a little more than 15 µm in the serial section study of megakaryocytes from an ITP patient.

b. The difference in true diameters between the smallest and the largest megakaryocytes in normal subjects will be no greater than in ITP since the diameters of sections of megakaryocytes were smaller in normal subjects than in ITP patients (fig.9).
On the other hand the observed difference in total megakaryocyte volume between ITP patients and control subjects was 90%. Therefore the influence of the difference in diameters of megakaryocytes between ITP patients and control subjects on the errors of the determination of total megakaryocyte volume can be neglected and it can be concluded that the observed large difference in total megakaryocyte volume between ITP patients and control subjects is a real difference.

c. The significance of the observed difference in the number of sections of megakaryocytes between ITP patients and controls.

An increase in diameter of megakaryocytes, results in an increase in the number of sections per megakaryocyte. In ITP patients the diameter of megakaryocytes was more than in control subjects. Therefore one has to consider whether the increased number of sections of megakaryocytes is the result of an increase in the diameter of megakaryocytes alone or also of an increase in the number of megakaryocytes.

In ITP patients the observed number of sections of megakaryocytes was 1.6 time more than in control subjects. So if one were to explain the difference in the number of sections between ITP and controls with a difference in true diameters alone, the mean true diameter of megakaryocytes in ITP must be about 1.6 time more than in control subjects. ($N = \frac{2R}{T} + 1$; see 2.1.1.). From the observed section diameters (fig.9) it is clear that the rather small difference in diameters of megakaryocytes between ITP and controls is not enough to explain the difference in the number of sections of megakaryocytes between ITP patients and control subjects.

So the increased number of sections of megakaryocytes in ITP patients in comparison with control subjects is the result of both an increase in the number of megakaryocytes and an increase in the diameters of individual megakaryocytes.

Conclusions

The observed increased total megakaryocyte volume in marrow biopsies of ITP patients in comparison with control subjects is not the result of a difference in errors, of the morphometrical method for total megakaryocyte volume determination, caused by the difference in diameters of megakaryocytes between the two examined groups. The larger diameter of megakaryocytes in ITP patients in comparison with control subjects can only partly explain the increased number of sections of megakaryocytes found in ITP patients. So the increased total megakaryocyte volume in ITP patients is caused by an increase in the number of megakaryocytes as well as by an increase in the mean size of the megakaryocytes.

The importance of the cellularity of the bone marrow for the evaluation of the total megakaryocyte volume and number of sections of megakaryocytes

The cellularity of histological sections varied greatly both in control subjects and, in patients with chronic ITP, or a bone marrow hypoplasia or myeloproliferative diseases (fig. 4). Since the number of haemopoietic cells per kg body weight in man under normal conditions is rather constant, this

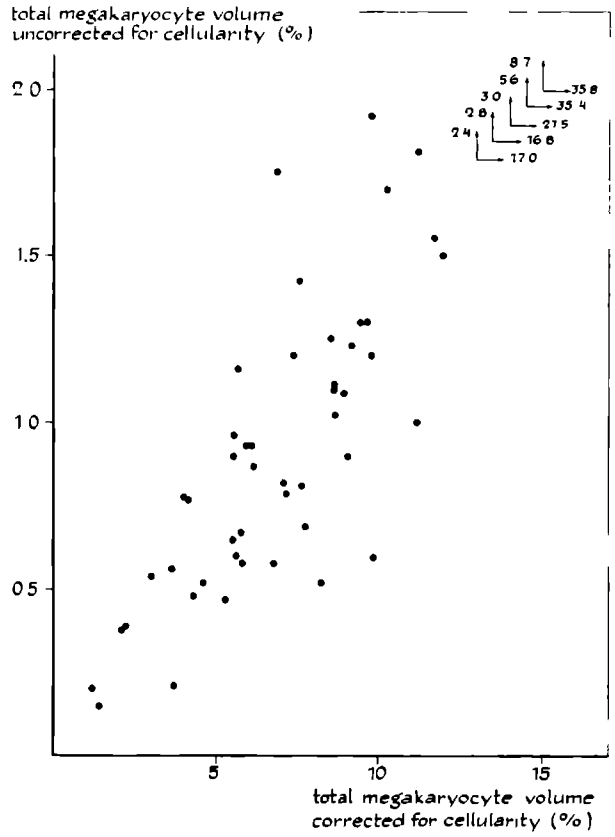

Fig. 19. Influences of cellularity of marrow biopsies on the estimation of the total megakaryocyte volume in normal contols and in patients with (a history of) thrombocytopenia or thrombocytosis. There is a direct relationship between the total megakaryocyte volume corrected for cellularity and that uncorrected for cellularity. The regression line is defined by $y = 0.1928 \times 0.3988$. The correlation coefficient is 0.936

means that the total bone marrow volume of the body varies considerably. Therefore a quantification of megakaryocytes, total megakaryocyte volume or megakaryocyte count, per unit marrow volume is unreliable unless a determination of the total bone marrow volume of the body is done. So it can be concluded that quantitation of megakaryocytes in marrow samples must be done in relation to a parameter for haemopoietic cellularity of the marrow sample. Otherwise the total megakaryocyte volume or the number of sections of megakaryocytes in histological sections can be over- or under estimated. In figs. 19 and 20 it can be seen that this over- or under estimation can be relevant.

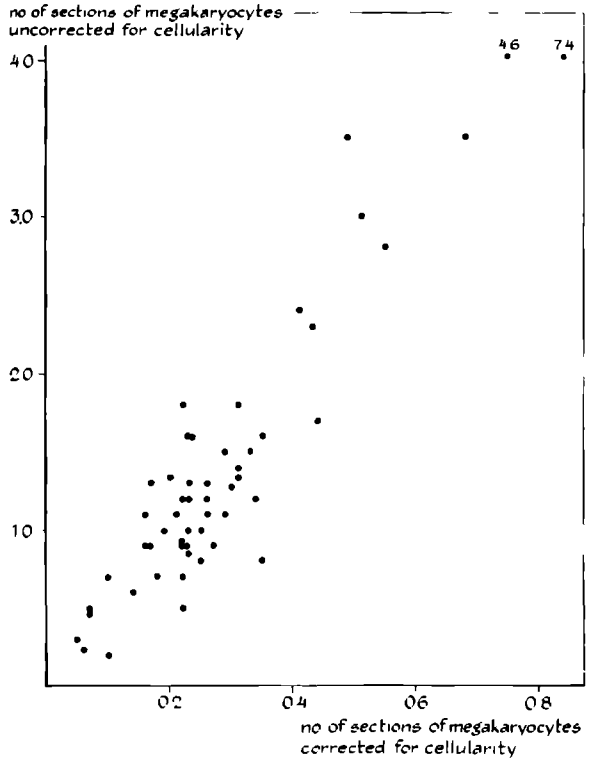

Fig.20.The influence of differences in cellularity of marrow biopsies on the megakaryocyte count in normal controls and in patients with (a history of) thrombocytopenia of thrombocytosis. In histological sections there is a direct relationship between the number of sections of megakaryocytes uncorrected for cellularity and corrected for cellularity. The cellularity is expressed as collective nuclear volume of non-megakaryocytic haemopoetic cells in proportion to the examined histological section volume. The quotient number of sections of megakaryocytes: number of test points on nuclei of non-megakaryocytic haemopoietic cells is plotted on the x axis. The regression line is defined by y= 5.63 x -0.20. The correlation coefficient is 0.889

Clinical value of the total megakaryocyte volume in marrow biopsies

With the results of the total megakaryocyte volume determinations, using the described morphometrical method, thrombocytopenia caused by an decreased platelet production, as in hypo-plastic anaemia, could be differentiated from thrombocytopenia through an increased destruction of platelets as in chronic ITP (fig. 8). Thrombocytopenias can also be differentiated by determination of platelet survival. However, determination of platelet survival is often not feasible in patients with a severe thrombocytopenia, at least not by

using autologous platelets. Platelet survival times determined using homologous platelets may be shortened by foregoing transfusions or pregnancies. If the determination of platelet survival is not feasible, or if the results are unreliable then determination of total megakaryocyte volume can be particularly useful for differentiating between hypoplastic thrombocytopenia and thrombocytopenias caused through increased platelet destruction or ineffective thrombocytopoiesis.

A normal platelet count in patients with a history of ITP can be the result of an increased platelet destruction together with an increased platelet production; a compensated thrombocytolysis. The newborns of mothers with such a compensated thrombocytolysis can have a severe thrombocytopenia probably due to the permeability of the placenta for antiplatelet antibodies from the mother. For obstetrical reasons it is important to know if the child has a thrombocytopenic bleeding tendency. This can be expected if the life span of platelets from the pregnant woman is shortened. However, determination of platelet life span with radioactive markers during pregnancy should not be carried out. In these cases of pregnancy with a normal platelet count but a history of chronic ITP, determination of total megakaryocyte volume in marrow biopsies proved to be useful for detection of compensated thrombocytolysis. Our observations of 3 pregnant women with normal platelet count during pregnancy suggests a relationship between the increase of the total megakaryocyte volume of the mother and the severity and duration of the haemorrhagic diathesis of the child. This suggests that the chance of neonatal cerebral bleeding as the result of delivery can be predicted from the result of determination of total megakaryocyte volume from the mother.

In patients with (a history of) ITP a stimulated thrombocytopoiesis was considered to be present 21 times since there was a thrombocytopenia, a shortened platelet life span or a child with a thrombocytopenia was born to the patient (chapter 3). In these cases the total megakaryocyte volume was increased in 16 times and normal 5 times. However, it is questionable if the total megakaryocyte volume was normal in spite of a stimulated thrombocytopoiesis since 4 of these 5 patients with a normal total megakaryocyte volume were splenectomized and normal values for total megakaryocyte volume in splenectomized subjects are unknown. It can not excluded that these values might be decreased in comparison with non-splenectomized subjects, since, as discussed in chapter 1, it is unknown if the regulatory mechanism for thrombocytopoiesis is directed toward constant platelet counts per kg body weight or per litre blood. If this feed-back mechanism is directed toward the platelet concentration of the blood,

this implies that after removal of the splenic platelet pool, the platelet production will decrease about 30%. If this is true then the total megakaryocyte volume of normal subjects after splenectomy will also decrease 30% even though blood platelet counts remain normal So the upper border of the normal range for total megakaryocyte volume corrected for cellularity will decrease from 7.8% before splenectomy to 5.5% after splenectomy. In the four splenectomized ITP patients with a stimulated thrombocytopoiesis the values for total megakaryocyte volume were between 5.5% and 7.8%, so they were perhaps somewhat increased. This would mean that in only one ITP patient (case no 7), in spite of a stimulated thrombocytopoiesis, the total megakaryocyte volume was not increased.

Comparison of the estimated total megakaryocyte volume with the literature

The principle of the method employed for determination of the total megakaryocyte volume in marrow biopsies corresponds with the method used by Lundin et al. (1972). They determined the number and the mean area of sections of megakaryocytes from histological sections in photographs. The normal mean area of sections of megakaryocytes (290 μm^2) was in accordance with our results. In normal subjects they found a total megakaryocyte volume of 20% less than in our study. Our values for total megakaryocyte volume in ITP patients agree in part with the findings of Harker (1970c). However in our study the increased total megakaryocyte volume was not found in all ITP patients and was less marked. The advantage of the method employed in this study for determination of the total megakaryocyte volume in marrow sections is that the product of the number of sections of megakaryocytes and their areas is estimated directly instead of by separate determinations of the number of sections and their diameters or areas.

The standard deviation of the total megakaryocyte volume in our control group is 22% of the mean value. In this respect the method used is nearly comparable with the isotope dilution method of Harker (1968) (SD 11%) and is preferable to the planimetric method of Lundin et al (1972) (SD 53%). The observed range in total megakaryocyte volume in normal subjects can not be caused by the method of determination but arises from the biological range in total megakaryocyte volume. The biological variation in total megakaryocyte volume can be expected since platelet count and platelet life span show some variation between normal subjects.

The number of sections of megakaryocytes in marrow biopsies of ITP patients found in this study was in the same range as reported by others (table 21).

Table 21

The number of megakaryocytes in bone marrow sections in patients with chronic ITP

Studies	Megakaryocyte no. of sections (x normal)		No. of patients	Platelet count ($x10^3/\mu l$)	Splenectomy
	mean	range			
Barta (1932)	1.0		1	10	
Lawrence and Kutti (1934)	<1	<1-1	6	**	
Nickerson and Sunderland (1937)	1.1	0.5-1.9	4	***	
Harker (1970)	3.0*	1.4-5.5	14	27 (4- 94)	before
	1.5*	1.1-3.8	7	195 (9-313)	after
This study	1.9	1.1-3.4	8	46 (7- 92)	before
	1.5	0.8-2.6	14	125 (2-322)	after

* number of whole megakaryocytes not the number of sections

**"markedly reduced"

*** "widespread petechiae"

Relationship between morphometrical determinations and platelet counts

In this study no relationship was found between total megakaryocyte volume and the peripheral platelet count in ITP patients (table 5). This confirmed the findings of Harker (1970c) and Kutti et al. (1973) in ITP patients. A relationship between platelet count and total megakaryocyte volume can only be expected if a) shortening of platelet life span is equal in all ITP patients, b) if platelet distribution between the blood and spleen is constant and c) if the total megakaryocyte volume is a parameter for effective platelet production. However platelet survival varies considerably in ITP patients. So a relationship between peripheral platelet count and total megakaryocyte volume cannot be expected. For the same reason, since there is a variable platelet destruction in ITP, one would not expect a relationship between the peripheral platelet count (which is the result of production and destruction)

and the mean megakaryocyte volume (which will be correlated with the production alone) as was observed by Harker (1969, 1970 c). In our study, this relationship was much less clear. It seems unlikely that the difference between our results and Harker's is caused by less acurate determinations of parameters of megakaryocyte size in this study, although the histological sections employed by us were thicker.

Megakaryocyte ploidy

In this study the percentage of megakaryocytes of 32 N and 64 N in control subjects was somewhat greater than in the literature (table 3). The ploidy values at which the frequency peaks of measured ploidy histograms were localized were a little greater than the 8N- 16N- 32N and 64N values as calculated with the arbitrary fluorescence light units of 2N metamyelocytes (fig.15). The cause and significance of this phenomenon is not clear. This difference cannot be due to the 2N reference nuclei employed. Metamyelocytes were used as 2N reference because they are easily recognizable and do not proliferate. We found that the Feulgen-DNA values of metamyelocytes were somewhat more than the values for polymorphonuclear leucocytes. This is in accordance with the observation of James (1973) who reported larger Feulgen-DNA values in cells with a looser chromatin meshwork than in cells with the same karyotype but with condensed chromatin. The validity of the method employed is supported by the results of ploidy values in 2 patients with chronic myeloid leukaemia and 1 patient with idiopathic thrombocytosis. In these patients respectively decreased and increased DNA values have been measured (Penington 1971c, Lagerlöf 1972; Harker et al.1969, Albrecht et al. 1974).

In this study an increase in ploidy values in patients with (a history of) chronic ITP was found. This cannot be explained by a selective loss of small 8N megakaryocytes in patients with ITP or a selective loss of large 64N, megakaryocytes in control subjects during the marrow sampling or the subsequent preparations, or by some selectivity during DNA measurements. Such selectivities cannot explain the observed shift of the peak frequency of the ploidy distribution from 16N in control subjects to 32N in patients with chronic ITP. The ploidy findings in this study of ITP patients are quite the contrary of the results of Queisser et al. (1971) and Paulus et al. (1973) (table 3). In our study the subjects examined were all patients with chronic ITP in a steady clinical state. It is possible in patients with acute ITP that during the first

days of the disease the stimulation of thrombocytopoiesis has already led to an increase in cells which differentiate from precursor cells into megakaryocytes, but that there has been insufficient time since the stimulation for the development of maximal ploidy values. In our patients with an acute thrombocytopenia due to cardiac surgery with extracorporal circulation there was also no increase in DNA values although there was a stimulated thrombocytopoiesis. The observed shift to higher ploidy values in patients with chronic ITP probably means that more megakaryocytes than normal reach a high ploidy level. Other explanations could be the premature death of some ploidy classes or a selective alteration in the maturation rate e.g. a prolonged maturation time of megakaryocytes of a high ploidy level or a shortened maturation time of megakaryocytes of a low ploidy level which would also result in an increase in the mean ploidy level.

2.3. CONCLUSIONS

The described morphometrical method for determination of the total megakaryocyte volume in bone marrow biopsies (the product of the number of megakaryocytes and their individual volumes) seems to be a good parameter for the potential platelet production capacity of the marrow. Thrombocytopenia caused by hypoproliferation of the marrow could be differentiated from other forms of thrombocytopenia. In patients with chronic ITP normal and increased values of the total megakaryocyte volume have been found.

When there was a normal platelet count in pregnant subjects with a history of chronic ITP, the total megakaryocyte volume of marrow biopsies proved to be valuable for predicting the platelet count of the newborn.

It was demonstrated that because of the differences in cellularity of the bone marrow sections between different subjects ,quantitation of megakaryocytes per unit marrow volume is less valuable than quantitation of megakaryocytes in relation to a parameter for cellularity of the bone marrow.

The estimation of the total megakaryocyte volume in histological marrow sections will be a parameter for platelet production when no abnormalities of platelet volume and megakaryocytopoiesis, such as extramedullary haematopoiesis, ineffective platelet production, changes in maturation- and platelet release velocity of megakaryocytes and very acute stimulation or suppression of platelet

production occur. Reports of other workers suggest that in practice the influence of these factors is often too small to lead to a discrepancy between the total megakaryocyte volume and platelet production. So the estimation of the total megakaryocyte volume in marrow biopsies can often be used instead of the estimation of platelet survival to gain an impression of platelet production.

In patients with chronic ITP the size and the polyploidy level of a part of the megakaryocytes was increased as well as the total megakaryocyte volume. An increase in megakaryocytic size and ploidy can be explained as an expression of a stimulated thrombocytopoiesis.

The increase in total megakaryocyte volume of ITP patients cannot be explained only by the increase in the volume of the individual megakaryocytes; the total number of megakaryocytes proved to be also increased.

CHAPTER 3

OBSERVATIONS ON SOME KINETIC ASPECTS OF MEGAKARYOCYTOPOIESIS AND THROMBOCYTOPOIESIS

As described in the foregoing chapter an increased total megakaryocyte volume was found in marrow biopsies from patients with chronic ITP. To understand the significance of this increased megakaryocyte mass for platelet production one requires information regarding the rate of turnover of megakaryocytes and the effectivity of platelet production. Up until now direct measurements of these parameters have not been possible. In order to gain an impression of the functional significance of the increased total megakaryocyte volume in ITP, a number of qualitative aspects of megakaryocytopoiesis have been studied; in particular DNA synthetic activity, maturation of megakaryocytes and the individual size of the platelets produced.
Moreover in a number of ITP patients the effective platelet production was calculated from the increase in blood platelet counts which occurred following splenectomy.

3.1. THYMIDINE, URIDINE AND METHIONINE UPTAKE BY MEGAKARYOCYTES MEASURED USING AUTORADIOGRAPHY

3.1.1. Introduction

As described in chapter I recent concepts concerning megakaryocytopoiesis are derived mainly from autoradiographic studies of megakaryocytes and their precursors using ^{3}H-thymidine, combined with determinations of the DNA content of megakaryocytes and the estimation of their proportional distribution in the various maturation stages.

In experimentally induced thrombocytopenia the following changes in megakaryocytopoiesis are often observed: there is an increase in the number of megakaryocytes, an increase in polyploidy and an increase in the percentage of mega-

karyocytes labelled with ^{3}H-thymidine (labelling index). In some studies changes in maturation of megakaryocytes and platelet release velocity have also been reported (Feinendegen et al. 1962; Ebbe et al. 1965, 1968a, b, 1970a; Odell et al. 1962, 1969; Rolovic et al. 1970a,b, Mac Pherson 1974). An increase in the percentage of megakaryocytes labelled with thymidine can be interpreted as:

1. an increased turnover of DNA synthesizing precursor cells in recognizable megakaryocytes
2. an increased polyploidization in recognizable megakaryocytes
3. a shortened megakaryocyte maturation time.

Labelling studies of megakaryocytopoiesis in experimentally induced immune thrombocytopenia gave conflicting results. Rolovic et al. (1970a) concluded that in this condition the bone marrow fails to react properly to the stimulus of the low platelet count. They suggested an injurious effect of platelet antibodies on megakaryocytes and therefore on platelet production. One of their arguments was that the initial value of the thymidine labelling index after induction of the thrombocytopenia in the immune thrombocytopenia group, was lower than the labelling index in the group with thrombocytopenia produced by thrombocytopheresis. However in similar experiments Odell et al.(1969) found evidence of a normal stimulation of megakaryocytopoiesis, similar to the labelling pattern observed after massive bleeding (Odell et al. 1962). The different results obtained in experimentally induced thrombocytopenia may be explained by differences in experimental procedures such as injection-time of thymidine or the degree of the thrombocytopenia induced (Odell et al. 1971). Besides that, it can be questioned whether or not these experimental conditions are comparable to pathological states in human beings.

Observations concerning ^{3}H-thymidine labelling of human megakaryocytes are scarce. Cronkite et al. (1961), Müller (1967) and Clarkson et al. (1971)studied human megakaryocytes after injection of ^{3}H-thymidine in some patients. From these studies it can be concluded that only immature megakaryocytes are labelled and the mean time needed for DNA synthesis and maturation of megakaryocytes together in man is probably about 5 days. This is longer than the period of 2-3 days observed in rats. Besides these in vivo studies there is one study of in vitro incubation of human megakaryocytes with ^{3}H-thymidine (Queisser et al. 1971). These authors observed no differences between the labelling indices obtained from three normal subjects and from three patients with ITP. The labelling index of megakaryocytes of type I (the most immature megakaryocytes)

varied from 32 to 54%.

It can be concluded that studies of DNA synthetic activity of megakaryocytes in man are scarce. Moreover the significance of the results is difficult to appreciate because the in vivo labelling studies were performed in leukaemic and comatose patients. On the other hand, knowledge of DNA synthesis proved to be very important in the development of recent concepts of megakaryocytopoiesis in animals. For these reasons we decided to study the ^{3}H-thymidine incorporation activity of megakaryocytes in man, particularly in patients with thrombocytopenia and in control subjects. Moreover some studies concerning the uptake of labelled uridine and methionine were performed to get an impression of ribonucleic-acid-(RNA) and protein synthesis in normal subjects and in ITP patients.

3.1.2. Materials and methods

Preparation of autoradiographs

^{3}H-thymidine

Bone marrow obtained by needle aspiration from the sternum or iliac crest was sampled in plastic tubes with a citrate anticoagulant as described (2.2.1). The marrow samples were incubated at 37°C for exactly one hour with tritiated thymidine (final concentration of 5 µC/ml). Tritiated thymidine was obtained from the Radiobiochemical Centre Amersham with a specific activity of 5 C per mmol. Smears of the marrow were made on glass slides, air-dried, fixed in methanol (99.5%) for 30 minutes and thereafter stained according to the May Grünwald-Giemsa technique (Merck). The stained smears were covered with polyvinylchloride (PVC) to prevent chemography of the photographic film emulsion and to conserve the morphology of the stained cells during the autoradiographic procedure. The principle of this method has been described by Keyser and Wijffels (1967). The stained smears were covered with a PVC layer by dipping the smears in a 15% PVC solution in 1 part of butyl-acetate and 2 parts of trichloroethylene with a semi-automatic dipping apparatus as described by Vrensen (1970) with a withdrawl velocity of 12 mm/min. The smears were air-dried at room temperature for 1 day. Subsequently the stained smears, covered with PVC, were coated with film emulsion by dipping the smears in liquid Ilford-L4 emulsion at a temperature of 32°C with the above mentioned dipping apparatus at a velocity of 84 mm/min. The autoradiographic exposure time was 7 days. During this time the preparations were stored

in the dark at 4°C. The autoradiographs were developed in Kodak D 19 b developer.

^{3}H-uridine and ^{3}H-methionine

The same procedure as described above was used in incorporation studies of ^{3}H-uridine or ^{3}H-methionine, both at final concentrations of 5 µC per ml bone marrow. Tritiated uridine and methionine were obtained from the Radiochemical Centre Amersham with specific activities of 30 C per mmol and 5 C per mmol respectively.

Morphological classification of megakaryocytes

Megakaryocytes were classified approximately according to Feinendegen et al. (1962):

a. Type I: Earliest recognizable forms, with an intensely basophilic cytoplasm, without or with only sparse granulation, with a high nuclear-cytoplasmic ratio and a nucleus showing granular chromatin.
b. Type II: More differentiated forms, showing transition to an azurophilic cytoplasm, with more cytoplasmic granulation, a decreased nuclear-cytoplasmic ratio and with less granular chromatin in the nucleus.
c. Type III: Further differentiated forms, with much cytoplasmic granulation, a low nuclear-cytoplasmic ratio, with coarse chromatin. This group also includes the most mature forms with pycnotic nuclei.
d. Naked nuclei: There is no cytoplasm visible or only pale coloured cytoplasm without granulations.

When the classification of the megakaryocytes was difficult, the amount of granulation and the colour of the cytoplasm was decisive for classification.

Evaluation of autoradiographs

The autoradiographs were evaluated by light microscopy, all by the same technical assistant. The number of labelled megakaryocytes was expressed as the percentage of the total number of megakaryocytes, or as the percentage of type I megakaryocytes which were labelled. This parameter will subsequently be termed "the labelling index of megakaryocytes". To prevent bias due to selection of megakaryocytes, all the megakaryocytes in a smear were examined. In parts of the marrow smears where chemography had occurred, it was difficult to see if megakaryocytes were labelled. These megakaryocytes were excluded not only for estimation of the labelling index but also for the morphological differentiation. A minimum of 100 megakaryocytes were analysed from each subject, with a mean

value of 250 megakaryocytes.

Examined subjects

^{3}H-Thymidine studies

Normal controls (10)

Twelve examinations were carried out in a group of 5 normal volunteers and 5 patients without blood disorders or without diseases with known altered megakaryocyto-or thrombocytopoiesis. Patient no. 76 with febris e.c.i. and patient no. 77 with bronchogenic carcinoma were in a steady clinical state (table 23).

Patients with chronic ITP (12)

Fifteen examinations were carried out in a group of 12 patients. This group contained patients before and after splenectomy. Seven of them were examined during treatment with corticosteroids. Four cases who were studied following splenectomy showed, at the time of marrow aspiration, a normal blood platelet level. One of them (no. 14) was examined three days after splenectomy, in which period the blood platelet level had increased from 40 to 188 x 10^{3}/µl (table 24).

Patients with postoperative thrombocytopenia (6). In four out of five patients who developed a thrombocytopenia after cardiac surgery for prostheses of valves, a marrow sample was taken on the second of third postoperative day. In one patient (no. 42) this was done on the seventh postoperative day. One patient (no. 43) who developed a thrombocytopenia after a massive blood loss during prosthetic hip surgery, was examined 2 days after the operation (table 25).

Patients with various kinds of histories of thrombocytopenia (4). Four of five examinations in four patients with a history of severe thrombocytopenia were carried out during a period with normal platelet counts. One patient (no. 46) developed thrombocytopenic purpura during a fever period. The marrow sample from this patient was obtained while there was a rapid increase in the platelet count four days after the first haemorrhagic manifestation (table 26).

^{3}H-Uridine and ^{3}H-Methionine-studies

The studies with ^{3}H-uridine and ^{3}H-methionine were made in bone marrow samples from 3 control subjects and 6 patients with chronic ITP during a thrombocytopenic phase (table 27-30).

3.1.3. Results

^{3}H-Thymidine autoradiography, as a parameter for DNA-synthesis

^{3}H-Thymidine autoradiography revealed almost exclusively the earliest recognizable forms of megakaryocytes, type I. Rarely was a naked nucleic megakaryocyte labelled. Megakaryocytes of type II and III were not labelled.

The reproducibility of the estimation of the labelling index
A variation in the final concentration of ^{3}H-thymidine from 5 µC/ml to 25 µC/ml marrow solution, did not influence the labelling index. Also dilution of the marrow cell concentration by a factor of 5, prolongation of the exposure time of the autoradiographs from 7 to 13 days and the absence or presence of 1 or 2 polyvinylchloride layers between the stained marrow smears and the photoemulsion layer, did not change the eventual labelling index.

Table 22

Reproducibility of the estimation of the frequency distribution of morphological types and ^{3}H-thymidine labelling indices in 5 subjects. Two marrow aspirates were taken at different times from each of 4 subjects

Case no	Marrow aspirate	Megakaryocytes (%)				L I of type I*
		type			naked	
		I	II	III	nuclei	
7	10-04-'72	17	12	57	15	34
	04-05-'72	20	11	62	7	34
	05-07-'72	20	11	64	5	28
8	14-01-'72	20	20	54	6	30
	19-06-'72	24	15	55	6	24
47	12-04-'72	21	12	58	9	29
	04-05-'72	19	7	60	20	26
62	10-03-'72	25	21	47	8	32
	12-04-'72	23	17	51	9	32
65	10-03-'72	24	15	50	11	34
	10-05-'72	20	8	61	11	31

*Labelling index of type I megakaryocytes (% labelled) after in vitro incubation with tritiated thymidine

In five subjects the labelling indices were investigated two or three times on different occasions. The results of the estimations of the labelling index in repeated marrow aspirates in these 5 subjects are given in table 22. As can be seen, the reproducibility of the labelling index estimation is rather good.

Table 23

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-thymidine labelling indices in 10 control subjects

Case	Sex	Age	Marrow aspirate	Diagnosis	Megakaryocytes (%)				L I of
					type			naked nuclei	type I ^{3}H-TdR*
					I	II	III		
62	m	36	10-03-'72	-	25	21	47	8	32
			12-04-'72		23	17	51	9	32
65	m	28	10-03-'72	-	24	15	50	11	34
			10-05-'72		20	8	61	11	31
69	m	36	16-05-'72	Gastritis?	21	14	59	7	28
70	f	16	13-06-'72	Anorexia nervosa	32	12	53	3	28
72	m	34	10-03-'72	-	45	13	37	4	29
73	f	58	24-04-'72	-	26	14	46	14	33
74	m	60	24-04-'72	Lungfibrosis	24	16	55	5	24
75	m	43	07-03-'72	-	25	15	52	8	21
76	m	14	07-09-'72	Febris e.c.i.	13	11	71	6	31
77	m	65	29-05-'72	Bronchogenic carcinoma	23	13	59	4	31
Mean (n=12) ± SD					25±8	14±3	53±9	8 ± 3	29.5±4

*Labelling index of type I megakaryocytes (% labelled) after in vitro incubation with tritiated thymidine

In twelve observations of ten control subjects the mean labelling index for megakaryocytes of type I was 29.5% ± 4 (1 SD) (table 23).

Table 24

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-thymidine labelling indices in 12 patients with (a history of) chronic ITP

Case no	Sex	Age	Marrow aspirate	Splenectomy	Prednison mg/day	Platelet count (x10^3/µl)	Megakaryocytes (%) type I	II	III	naked nuclei	L I of type I ^{3}H-TdR*
1	m	41	06-07-72	-	-	16	22	15	60	3	21
5	f	55	28-07-72	-	-	38	23	15	54	8	31
6	m	59	04-09-72	-	60	29	39	20	38	3	28
7	f	65	10-04-72	-	-	16	17	12	57	15	34
			04-05-72	-	25	56	20	11	62	7	34
			05-07-72	+	10	184	20	11	64	5	28
8	m	26	14-01-72	+	40	94	20	20	54	6	30
			19-06-72	+	20	2	24	15	55	6	24
10	f	23	10-04-72	+	-	220	26	11	40	23	26
12	f	45	17-04-72	+	5	16	34	11	44	11	31
13	f	65	13-03-72	+	5	14	20	13	54	12	28
14	m	30	03-03-73	+	80	188	13	12	74	1	16
19	m	13	28-09-72	-	-	20	17	7	74	3	29
20	f	20	05-06-72	-	15	80	26	14	53	7	37
21	f	60	30-03-73	+	-	262	18	12	46	24	29
Mean (n=15) ± SD						82±87	23±7	13±3	55±11	9±7	28±5
Normal (n=12)± SD						208±50	25±8	14±3	53±9	8±3	29.5±4

*Labelling index of type I megakaryocytes (% labelled) after in vitro incubation with titriated thymidine

Table 24 shows that the mean percentage of labelled megakaryocytes in twelve ITP patients was in the same range as in normal subjects: 28% ± 5 (1 SD). Only one patient (no.14) examined 3 days after splenectomy, in which period the blood platelet count rose to normal, showed a rather low labelling index of

16%, while one other patient (no. 1) showed a boderline value. In the chronic ITP patients there was no relation between the labelling index and the blood platelet level and no difference between the labelling indices before and after splenectomy.

Table 25

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-thymidine labelling indices in 6 patients with postoperative thrombocytopenia

Case no	Sex	Age	Marrow aspirate	Diagnosis	Platelet count (x10^{3}/µl)	Megakaryocytes (%)				L I of type I ^{3}H-TdR*
						type			naked nuclei	
						I	II	III		
38	m	43	02-03-73	A.S.D.	83	42	12	45	1	57
39	m	49	29-03-73	A.I.	80	26	35	39	0	48
40	m	30	29-03-73	A.I.***	64	32	22	46	0	34
41	f	59	01-12-72	A.S.D.**	70	54	4	29	13	54
42	f	50	28-11-72	M.I.****	78	38	10	46	6	53
43	f	65	06-12-72	Prosthetic hip	66	35	14	51	0	43
Mean (n= 6) ± SD						38±10	16±11	43±8	3±5	48±8
Normal (n=12) ± SD						25±8	14±3	53±9	8±3	29 5±4

*Labelling index of type I megakaryocytes (% labelled) after in vitro incubation with tritiated thymidine

A.S.D.:atrial septal defect, * A.I.:aortic insufficiency, ****M.I.:mitral insufficiency

As is demonstrated in table 25 a high labelling index was observed in acute postoperative thrombocytopenia with a mean value of 48% ± 8 (1 SD) for type I megakaryocytes. The labelling indices of these acute forms of thrombocytopenia differed significantly from the labelling indices in normal subjects and

in patients with chronic ITP (Wilcoxon's two sample test; p=0.001) or patients with other kinds of thrombocytopenia (p=0.02) (fig. 21).

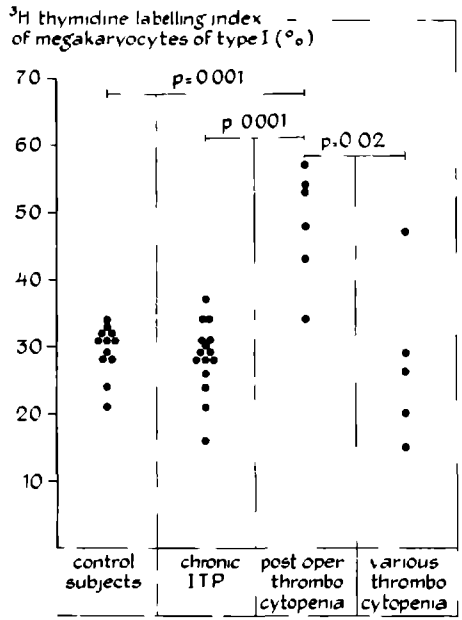

Fig. 21. Labelling index of megakaryocytes (type I) incubated with ^{3}H-thymidine, (10 control subjects, 12 patients with a history of chronic ITP, 6 patients with postoperative thrombocytopenia and 5 patients with various kinds of histories of thrombocytopenia). The labelling index in patients with acute postoperative thrombocytopenia is significantly increased in comparison with control subjects and patients with chronic ITP (p=0.001).

Table 26 shows the labelling indices in five patients with various kinds of histories of thrombocytopenia. Two of these patients showed normal values, one patient showed a low value. One patient (no.46) who had just recovered from an acute thrombocytopenic period, showed a high labelling index of 47%, comparable with the high labelling in the postoperative thrombocytopenic group.

^{3}H-Uridine

Labelling of megakaryocytes after in vitro incubation with ^{3}H-uridine was seen in all types of megakaryocytes with a predominance of the immature megakaryocytes.

Table 26

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-thymidine labelling indices in 4 patients with various kinds of histories of thrombocytopenia

Case no	Sex	Age	Marrow aspirate	Diagnosis	Platelet count (x10^{3}/µl)	Megakaryocytes (%) type I	type II	type III	naked nuclei	L I of type I ^{3}H-TdR*
44	m	23	23-02-72	auto-immune hemolytic anaemia, splenomegaly	124	22	9	57	11	15
45	f	32	14-04-72	lupus erythematosus	80	20	8	46	26	20
46	m	60	19-04-72	infectious thrombocytopenia	152	29	12	50	9	47
47	m	17	12-04-72	thrombotic thrombocytopenic purpura?	100	21	12	58	9	29
			04-05-72		309	19	7	60	20	26
Normal (n=12) ± SD						25± 8	14±3	53±9	8±3	29.5±4

*Labelling index of type I megakaryocytes (% labelled) after in vitro incubation with tritiated thymidine

Moreover, as is demonstrated in table 27 and table 28, the results were similar in control subjects and in ITP patients. Therefore, although the number of observations is rather small, no further investigations in this direction were performed. The significance of the high labelling index of megakaryocytes of the naked nuclei type observed in some ITP patients is very dubious because the absolute numbers of megakaryocytes of this type were very small.

Table 28

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-uridine labelling indices in 6 patients with chronic ITP

Case no	Sex	Age	Marrow aspirate	Splenec-tomy	Prednison mg/day	Platelet count (x10^{3}/ul)	Megakaryocytes				Labelling index*			
							type			naked nuclei	type			naked nuclei
							I	II	III		I	II	III	
1	m	41	06-07-72	-	-	16	22	17	55	6	50	16	5	21
5	f	55	28-07-72	-	-	38	20	14	60	6	50	16	6	0
6	m	59	04-09-72	-	60	29	35	13	48	4	49	8	5	10
8	m	26	14-01-72	+	40	94	32	13	40	14	74	15	3	27
19	m	13	28-09-72	-	-	20	27	12	57	4	43	0	2	0
20	f	20	05-06-72	-	15	80	19	14	61	7	66	12	1	24

* Labelling index of megakaryocytes (% labelled) after in vitro incubation with ^{3}H-uridine

Table 27

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-uridine labelling indices in 3 control subjects

Case no	Sex	Age	Marrow aspirate	Diagnosis	Megakaryocytes (%)				Labelling index*			
					type			naked	type			naked
					I	II	III	nuclei	I	II	III	nuclei
62	m	37	04-07-73	-	16	14	70	0	50	6	2	0
67	m	29	16-10-72	-	35	15	49	1	46	11	0	0
78	m	25	22-09-72	-	22	14	59	5	47	19	3	0

*Labelling index of megakaryocytes (% labelled) after in vitro incubation with tritiated uridine

^{3}H -*Methionine*

In a pilot study concerning autoradiography after ^{3}H-methionine incorporation, no differences were found between labelling indices in three control subjects and six patients with chronic ITP as is shown in table 29 and 30.

Table 29

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-methionine labelling indices of megakaryocytes in 3 control subjects

Case no	Sex	Age	Marrow aspirate	Diagnosis	Megakaryocytes (%)				Labelling index*			
					type			naked	type			naked
					I	II	III	nuclei	I	II	III	nuclei
70	f	16	13-06-72	anorexia nervosa	40	20	37	3	92	84	56	0
77	m	65	29-05-72	bronchogenic carcinoma	32	15	46	8	87	69	65	0
78	m	25	22-09-72	-	32	11	56	1	88	73	59	0

*Labelling index of megakaryocytes (% labelled) after in vitro incubation with tritiated methionine

Table 30

Percentages of megakaryocytes in various morphological maturation classes and ^{3}H-methionine labelling indices in 6 patients with chronic ITP

Case no	Sex	Age	Marrow aspirate	Splenec-tomy	Prednison mg/day	Platelet count (x10^{3}/µl)	Megakaryocytes (%)				Labelling index*			
							type			naked nuclei	type			naked nuclei
							I	II	III		I	II	III	
1	m	41	06-07-72	-	-	16	27	10	62	1	81	61	65	0
5	f	55	28-07-72	-	-	38	13	19	68	1	50	29	29	0
6	m	59	04-09-72	-	60	29	40	16	42	2	66	52	41	0
8	m	26	19-06-72	+	20	2	29	21	47	3	83	64	59	0
19	m	13	28-09-72	-	-	20	25	12	63	1	86	67	59	0
20	f	20	05-06-72	-	15	80	29	15	52	4	93	65	70	3

*Labelling index of megakaryocytes (%labelled) after in vitro incubation with ^{3}H-methionine

3.1.4. Discussion

Thymidine labelling-maturation stage

Labelling of megakaryocytes was only seen in type I and in some naked nuclei. Labelling of "naked nuclei" must be explained by the loss of cytoplasm by megakaryocytes of type I during the autoradiographic procedure. From animal studies Odell et al. (1968) also concluded that only the most immature megakaryocytes are able to synthesize DNA. However, in other investigations, polyploidization of megakaryocytes was also seen in less immature types (Ebbe et al. 1965; Müller 1967). This discrepancy may be caused by differences in criteria for maturation of megakaryocytes.

Thymidine labelling indices of megakaryocytes were expressed as percentages of labelled megakaryocytes of type I and not as percentages of all megakaryocytes for the following reason:

In areas with chemography which occurred mainly at the periphery of the marrow smear, it was difficult to see if megakaryocytes were labelled or not. Therefore megakaryocytes in areas with chemography were excluded. However, large megakaryocytes of type III which are more frequently seen at the periphery of the marrow film will be more often excluded than smaller, immature, types of megakaryocytes. As mature types are unlabelled this selective exclusion will result in an overestimation of the labelling index of megakaryocytes of all types. The chance that labelled and unlabelled megakaryocytes of type I will lie in areas with chemography will be about equal. So for megakaryocytes of type I there is no selective exclusion of labelled or unlabelled cells and their labelling index is to be preferred above the use of a labelling index that includs all types of megakaryocytes together.

Normal thymidine labelling in stimulated thrombocytopoiesis

No difference was found between the labelling index of megakaryocytes incubated with ^{3}H-thymidine in control subjects and chronic ITP patients, suggesting a normal DNA synthetic activity of megakaryocytes in ITP patients. This result seems to conflict with the observations in experimentally induced thrombocytopenia in which an increased thymidine labelling index of the megakaryocytes

was observed (Odell et al. 1969). This contrast may be explained by a difference in duration of the stimulation of thrombopoiesis: acute stimulation will result in an increased influx of megakaryocytes from the unrecognizable precursor pool. Feinendegen et al. (1962) observed that these precursor cells have a high DNA synthetic activity. Thus an increased influx of precursor cells into the recognizable megakaryocyte compartment will at first result in a high labelling index of the megakaryocytes. After some time the amount of megakaryocytes in the more mature megakaryocyte compartments will also increase and a new steady state with a normal labelling index will be obtained. This explanation of the normal thymidine labelling index of megakaryocytes in patients with chronic ITP, although one might expect a stimulated thrombopoiesis, is supported by the observations in this study in cases with an acute stimulation of thrombocytopoiesis. In 5 out of 6 patients with an acute severe thrombocytopenia following an operation, the labelling index of megakaryocytes of type I was greater than in 31 out of 32 examinations in control subjects and various kinds of patients (fig. 21). Only the patient recovering from an acute infectious thrombocytopenia (no. 46), also showed a high labelling index. The increased influx of precursor cells with a high DNA synthetic activity into the recognizable megakaryocyte compartment can explain the high labelling index of megakaryocytes of type I in cases with an acute stimulation of thrombopoiesis. Otherwise an increase in endomitotic activity in type I or an increase in the maturation velocity from type I to type II will also result in an increased labelling index of megakaryocytes of type I. The normal labelling index of megakaryocytes of type I in cases of chronic ITP suggests that in megakaryocytes of type I the quotient between the time spend in DNA synthesis activity and the rest of the time spend in stage I is unchanged. If in megakaryocytes of type I this time, not spent in DNA synthesis, is not absolutely increased then this implies that there is no extra time available for polyploidization in stage I. On the other hand we observed an increased DNA content in megakaryocytes from chronic ITP patients. At first sight this suggests that in chronic ITP patients megakaryocytes which are coming from the precursor pool into the recognizable compartment have already obtained an increased DNA content in the precursor pool. However the increased mean DNA value in ITP patients seems to be the result of an extra-nuclear replication of about 10% of the megakaryocytes or their precursors (table 18). Even if this occurs in megakaryocytes of type I it is questionable if this rather small percentage can be detected with the methods used in this study.

The observed thymidine labelling index of megakaryocytes was in accordance with the results obtained by Queisser et al. (1971) in 3 normal cases and 3 chronic ITP patients and with the labelling index of megakaryocytes of all types together of about 12%, after in vitro incubation, observed by Clarkson et al. (1971) in two leukaemic patients. Comparison of the labelling indices of immature types observed by Clarkson et al. (1971) and Queisser et al. (1971) and the results of this study is not possible due to differences in morphological classification criteria.

Uridine, methionine labelling

Although uridine and methionine used as markers for ribonucleic-acid-and protein synthesis are less specific than thymidine as a marker for DNA synthesis and the number of experiments carried out with these markers were small, the similar results obtained in patients with ITP and control subjects suggest that there are no important differences in ribonucleic-acid- and protein synthesis.

3.2. MATURATION OF MEGAKARYOCYTES STUDIED BY MORPHOLOGICAL DIFFERENTIATION OF MEGAKARYOCYTES

3.2.1. Introduction

Megakaryocyte differential counts give some information about megakaryocyte kinetics, especially when studied together with autoradiography. For instance an increase in the percentage of immature megakaryocytes can be interpreted as:

1. an absolute increase in megakaryocytes of type I
2. a longer duration of megakaryocytes in the type I compartment; this may be the consequence of an increase in polyploidization
3. a decrease in megakaryocytes of type II and III; this may be the result of premature cell death
4. a shorter duration of megakaryocytes in type II and III compartments as a result of an accelerated maturation time.

In most animal studies experimentally induced thrombocytopenia results in an increase in immature megakaryocytes after 24 hours (Craddock et al. 1955; Odell et al. 1962, 1969; Krizsa et al. 1968; Rolovic et al. 1970a). In some

studies these changes in the megakaryocyte differential count proved to be the result of an absolute increase in the number of megakaryocytes of type I while in other studies an accelerated maturation of more mature megakaryocyte was a contributory factor. Ebbe et al(1968a,b) observed a less obvious increase in the number of megakaryocytes of type I after acute stimulation and suggested that this was the result of a balance between an increased influx from precursor cells into the visible megakaryocyte compartment with an accelerated maturation time of the visible megakaryocytes. Megakaryocyte differential counts after prolonged depletion of platelets, comparable with chronic ITP, have scarcely been studied at all. Rolovic et al. (1970b) found a normal differential count in rats after a six week period of stimulated thrombocytopoiesis. This suggests a new steady state with an increased megakaryocyte turnover and eventually an increased maturation time. The duration of stimulation may be important for the results of morphological differential counts and may explain the different results found by the various investigators. Probably this problem is analogous to the discrepancy in findings concerning the megakaryocytic size after different periods of stimulation. After acute depletion of platelets the increase in size of megakaryocytes reported by Harker (1968b) and Ebbe et al. (1968a) was less obvious than after prolonged depletion of platelets (Pennington et al. (1970).

Changes in proportional distribution of the various maturation stages and morphological abnormalities of megakaryocytes have been described in patients with ITP. A recent review is given by Rebuch et al. (1970). The reported observations are conflicting. This can be explained partly by differences in the classificiations of thrombocytopenic patients, especially in the older literature. Some megakaryocytic features reported in ITP are: a loss of platelet formation, a reduction in azurophilic granulation, vacuolization and coarsening of chromatin nuclear pattern. These qualitative alterations are almost undocumented quantitatively and in fact in our experience their existence is very doubtful.

There are some well documented studies concerning the frequency distribution of megakaryocytic types in ITP patients. Diggs et al. (1948) showed a shift to immature megakaryocytes in 22 patients with ITP. However these observations were carried out in the acute stage of the disease. Presley et al. (1952), using well formulated criteria for maturity of megakaryocytes, studied acute and chronic ITP patients and also found an increased relative immaturity of megakaryocytes. In recent studies Queisser et al. (1971) reported a normal megakaryocyte differentiation in ITP while Harker (1970c) observed a decreased cyto-

plasmic granulation, indicating a relative predominance of immature megakaryocytes, only in chronic ITP patients with platelet counts of less than 20,000/µl. In chronic ITP patients with platelet counts higher than 40,000/µl Harker found that the pattern of cytoplasmic granulation was not different from normal. Recent cytochemical studies by Kass (1973) showed that the ITP megakaryocytes are enzymatically young when compared to normal megakaryocytes. Albrecht et al. (1974) described megakaryocytes in ITP, which were larger, more basophilic and which occasionally showed platelet formation in basophilic megakaryocytes.

3.2.2. Materials and methods

Criteria for morphological classification of May Grunwald-Giemsa stained megakaryocytes are described under 3.1.2. Differential counts of megakaryocytes were carried out on autoradiographs of marrow smears from control subjects and the patients which were studied for thymidine incorporation of megakaryocytes. A minimum of one hundred megakaryocytes was differentiated.

3.2.3. Results

The reproducibility of the determinations of the distribution of megakaryocytes in their various maturation classes in repeated marrow aspirates in 5 subjects is shown in table 22.

The relationship between the labelling index of megakaryocytes incubated with ^{3}H-thymidine and the percentage of megakaryocytes of type I is given in figure 24.
The proportional distribution of megakaryocytes in their various maturation stages in control subjects is given in fig. 22 and table 23. Most megakaryocytes were classified as mature, type III, megakaryocytes.

In 15 examinations of 12 patients with (a history of) chronic ITP the frequency distribution of megakaryocytes did not deviate from normal (table 24). There was no relationship between the percentage immature, type I, megakaryocytes and the platelet count in ITP patients. Megakaryocyte differential counts before and after splenectomy in ITP patients showed no essential differences.

In the patients with acute thrombocytopenia following cardiac surgery and massive bleeding, a predominance of immature megakaryocytes was observed (table 25).

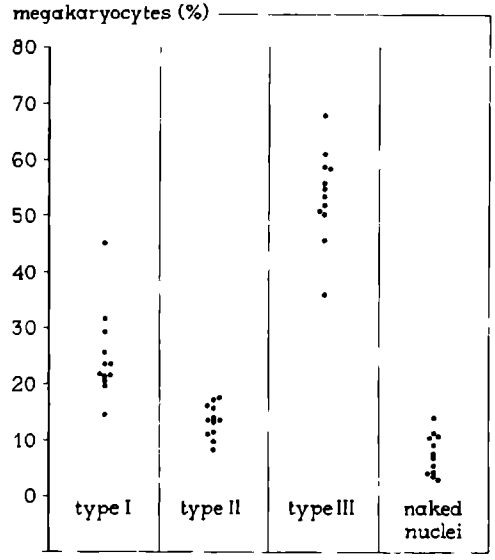

Fig.22.Frequency distribution of megakaryocytes in morphological maturation classes from normal control subjects

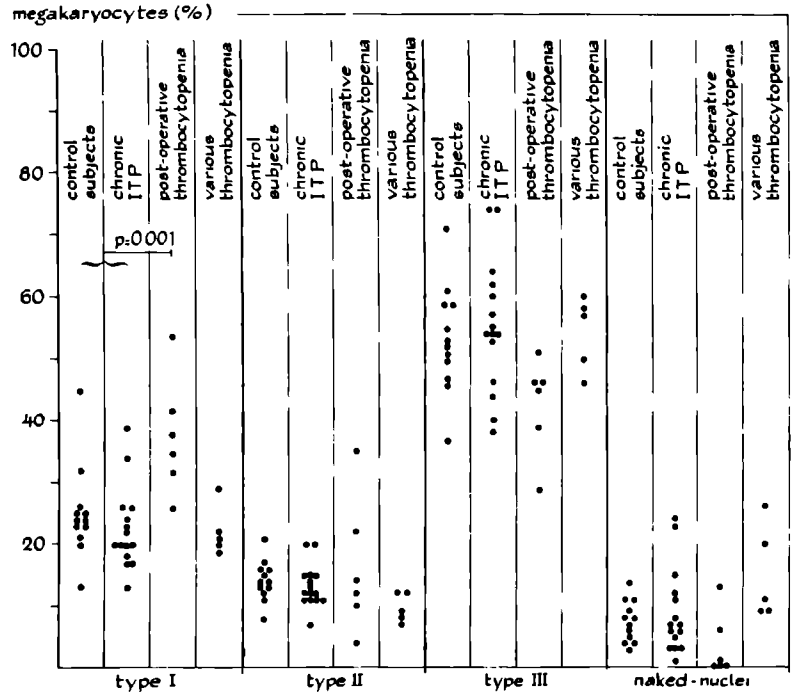

Fig.23.Morphological analysis of megakaryocytes classified approximately according to Feinendegen et al. (1962) (10 control subjects, 12 patients with a history of chronic ITP, 6 patients with acute postoperative thrombocytopenia and 5 patients with various kinds of histories of thrombocytopenia)

The mean percentage of type I megakaryocytes in the post-operative thrombocytopenia group was 38%, in contrast to values of 25% and 23% in control subjects and patients with chronic ITP (fig. 23). These differences are significant, p=0.001, according to Wilcoxon's two sample test.

In the patients with various other kinds of thrombocytopenia a normal differential count was observed (table 26).Patient no. 46, who was just recovering from an acute thrombocytopenia, also showed a normal percentage of type I megakaryocytes

3.2.4. Discussion

Although any classification of megakaryocytes into their various maturation classes is arbitrary and subjective, the results of this study in control subjects agrees with other studies using megakaryocyte differential counts according to the criteria of Feinendegen et al. (1962) (Queisser et al. 1971; Ebbe et al. 1965).

As is many animal studies of experimentally induced acute thrombocytopenia we observed a predominance of immature megakaryocytes in patients with acute stimulation of thrombocytopoiesis produced by thrombocytopenia following cardiac surgery or massive bleeding. This suggests an increased influx of megakaryocytes into the visible megakaryocyte compartment or a relatively longer retention of megakaryocytes in the type I compartment. In chronic ITP patients no increase in type I megakaryocytes was observed in this study, suggesting a normal maturation time of megakaryocytes or an equal increase in the maturation time of all maturation stages, compensated by an increased polyploidization time of megakaryocytes in the type I compartment. The percentage of megakaryocytes without cytoplasm (naked nuclei) varied from 1 to 26. This variation was more than the variation in the percentages of other types of megakaryocytes. Moreover, sometimes thymidine labelling of naked nuclei was observed while megakaryocytes of type II and type III were never labelled in this study. Naked nuclei represent not only the final stage of degenerating megakaryocytes but also megakaryocytes which are damaged during the aspiration of the marrow sample, during the incubation period with the radioactive material or in the preparation of the marrow film. For this reason the relation between the percentages of megakaryocytes of types I, II and III are more important than the percentages of

naked nuclei. In some cases the number of megakaryocytes of type III will be decreased by selective accumulation of type III cells in the tail of the marrow film, which areas because of chemography are sometimes excluded from evaluation.

Some positive correlation between an increased ^{3}H-thymidine labelling index of megakaryocytes of type I and an increased percentage of megakaryocytes of type I was observed in patients with postoperative thrombocytopenia (fig. 24). This suggests an increased influx of megakaryocytes from the unrecognizable precursor compartment, an increased endoduplication of the nucleus of megakaryocytes of type I, or both.

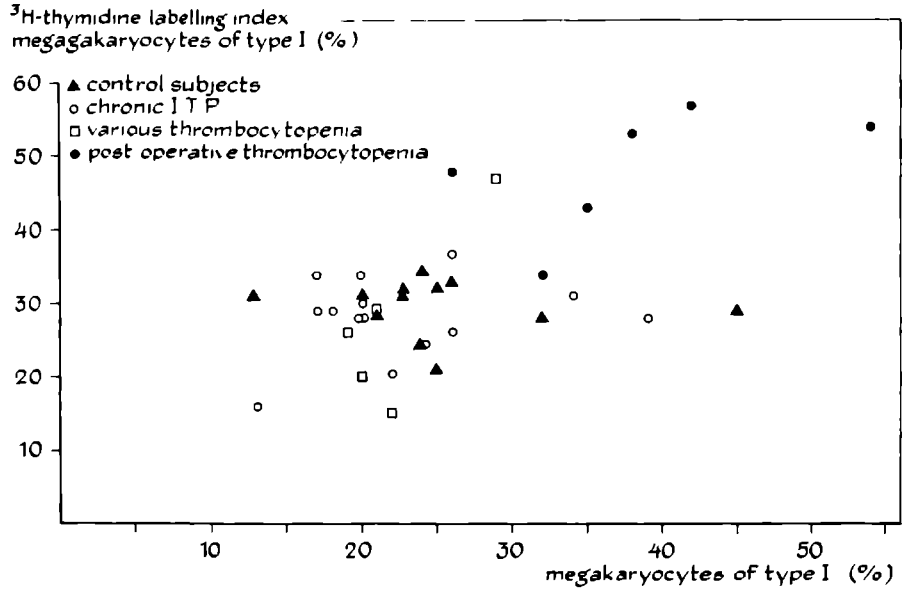

Fig. 24. Relationship between percentage of megakaryocytes of type I and their labelling index after incubation with ^{3}H-thymidine (10 control subjects, 12 patients with a history of chronic ITP, 6 patients with acute postoperative thrombocytopenia and 5 patients with various kinds of histories of thrombocytopenia). The regression line is defined by Y=0.65 x + 15. The correlation coefficient is 0.608.

3.3. THROMBOCYTOPOIETIC ACTIVITY INDICATED BY PLATELETS SIZE DISTRIBUTION

3.3.1. Introduction

Platelet size can be determined visually on blood smears by measurements of diameters of platelet areas and by electronic volume-plotting of platelet suspensions. The results of the different techniques agree quite well (Garg et al. 1971, 1972; Murphy et al. 1972; Kraytman 1973; Paulus 1974). Normal platelet volumes vary from about 5 to 9 μ^3. Until recently it was accepted that the size of platelets diminishes during their ageing in the peripheral circulation. The log normal platelet volume distribution curve, observed with different techniques, has been explained on the basis of age distribution (Mc Donald et al. 1964; Bull et al. 1965; Goguel et al. 1973). However, recently Paulus (1974) provided data against an age-dependent platelet volume. He observed a log normal volume distribution in a platelet population with an age of some hours. Moreover Paulus (1974) found no decrease in the volume of these young platelets during their life span when they were transfused into patients lacking platelet production. He concluded that platelet volume distribution was not age-dependent but was the result of the development of platelets in megakaryocytes. Paulus (1974) suggested that growth rate and fragmentation of the megakaryocytic cytoplasm are decisive for the genesis of the volume distribution of platelets.

An abnormal platelet size distribution, resulting in an increased percentage of large platelets (megathrombocytes) is observed in patients with a shortened platelet life span, such as patients recovering from consumptive thrombocytopenia, some hereditary platelet disorders and in some haematological diseases with increased and normal platelet turnover. (Kurstjens et al. 1968; Falcao et al. 1971; Saarni et al. 1971; Baadenhuysen 1971; Karpatkin et al. 1971; Garg et al. 1972; Godwin et al. 1972; Murphy et al. 1972; Paulus 1974). In all cases (normal or abnormal thrombocytopoiesis) a log normal distribution of platelet volumes was observed.

In ITP patients an increased percentage of large platelets was observed not only in thrombocytopenic patients but also in patients with a history of ITP with a normal platelet count but a decreased platelet survival (Garg et al. 1971, 1972; Murphy et al. 1972;Paulus 1974). This suggests the usefulness of the percentage of the large platelets as an index for a stimulated thrombocytopoiesis. Moreover the positive correlation between the percentage of large platelets and the number of megakaryocytes found in various kinds of thrombocytopenia and non-thrombocytopenic disorders with shortened platelet survival,

suggests the usefulness of the percentage of large platelets as an index for the degree of platelet production (Garg et al. 1971). However there was no positive correlation between the percentage of large platelets and the number of megakaryocytes in cases with thrombocytotic disorders and megaloblastic anaemias.

So in ITP the significance of an increased percentage of large platelets can be:

1. an indication of an increased platelet production
2. an indication of an abnormal thrombocytopoiesis
3. both

Some arguments can be provided for the assumption that the abnormal platelet size distribution with an increase in large platelets in ITP indicated an increased platelet production:

a. Cases of ITP with a shortened platelet survival but a normal platelet count caused by compensated, increased platelet production, show an increased percentage of large platelets

b. During successful treatment with corticosteroids or after splenectomy a rapid decrease in the percentage of large platelets occurrs in combination with a prolongation of platelet life span, although antiplatelet antibodies, which could possibly be deleterious for megakaryocytes, are still present.

In this study platelet size distribution curves were investigated in ITP patients in order to look for signs of stimulated thrombocytopoiesis and correlations between platelet volumes and other measured characteristics of megakaryocytopoiesis.

3.3.2. Materials and methods

Measurement of platelet size

Preparation of platelet suspensions

Venous blood was collected in plastic tubes and anticoagulated with ethylene diamine tetra-acetate (Samama et al. 1973). Platelet rich plasma was obtained by centrifugation at room temperature (2.2 L). A sample of 10 µl platelet rich plasma was diluted in 10 ml Isoton (Coulter electronics, Bedfordshire, England).

Platelet size measurements were performed within 2 hours after blood sampling.

size distribution was plotted with a Coulter model P 64 plotter, full scale 4096, attached to a Coulter counter, model ZF. A 50 μ aperture tube was used. The machine settings were as follows: Sensitivity 0.707 or 1.0: Controls (aperture) 8 and Treshold 10. The calibration was performed with Coulter latex particles of 3.42 μ^3 volume. Those particles showed a frequency peak in channel 14 with a sensitivity setting of 0.707. Each Treshold division was equal to 0.244 μ^3. So particle plots from channel 1 to 64 encomposed volumes of 0.25 to 15.62 μ^3.

Analysis of platelet size curves

When the platelets were classified in log volume classes the frequency distribution was symmetrical (fig. 25). Each frequency distribution was characterized by:

a. the mode of the ^{e}log volume distribution (μ), indicating the peak frequency
b. the standard deviation of the mode of the ^{e}log volume (σ) indicating the variation in platelet size

The mean platelet volume could be calculated from the formula

$$\text{mean platelet volume} = \exp.\ (\mu + 0.5\sigma^2) \qquad (14)$$

In some cases the largest platelets could not be visualized even at low amplification. In those cases the invisible part of the frequency distribution curve was calculated with an IBM computer from the available part of the curve assuming a log normal distribution. In some measurements a discordance between the measured frequency distribution and the expected log-normal curve distribution was found, caused by electrical background noise in the first channel. In those cases the measurement results of the first channels were discarded.

Examined subjects

Control subjects (21) were volunteers, mostly healthy laboratory workers and some patients without blood disorders. Of 15 patients with (a history of) chronic ITP 12 were studied after splenectomy. Most patients were in apparent remisson. In only 5 out of 17 examinations in 15 patients was the platelet count less than 100,000/μl at the time of examination.

3.3.3. Results

Platelet volume distribution proved to be log normal (fig. 25).

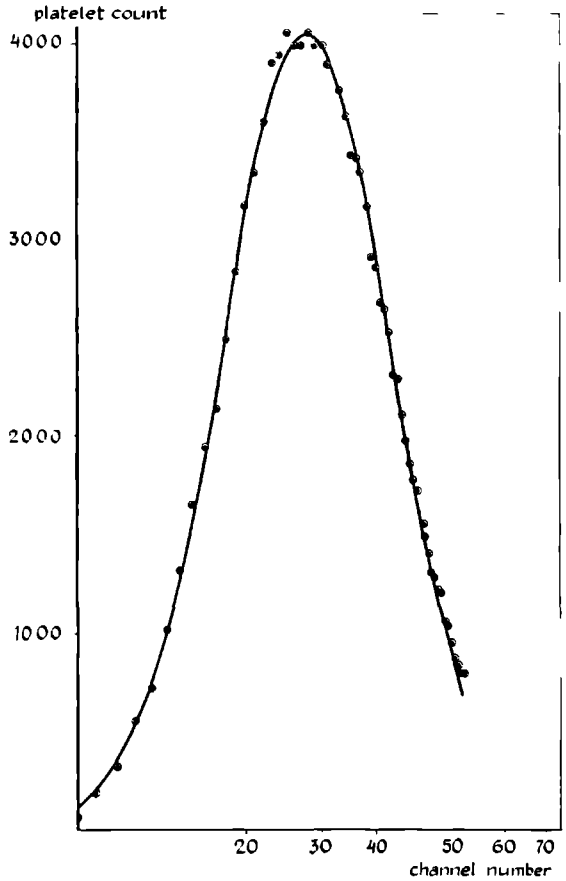

Fig. 25.Platelet volume distribution in a patient with chronic ITP. Platelets are classified in log volumes classes. The number on the abcis indicates the channel number of the coulter plotter. The number of platelets counted in each volume class is indicated by ◉. The curve is the log normal distribution curve which, using an IBM computer, proved to correspond best with the measured data.

In control subjects the mean platelet volume was 6.83 μ^3 ± 0.85 (1 SD) (table 31). Ten out of the seventeen examined patients with (a history of) chronic ITP showed mean platelet volumes within the normal range (mean ± 2 SD). Seven ITP patients showed an increased mean platelet volume and an increased mode log volume (table 32, fig. 26). In two of these seven patients the variation in platelet size, expressed as the standard deviation of log volumes of platelets, was increased. One patient (no.9), with a normal mean platelet volume, showed an increased variation in platelet size. The difference in the mean platelet volume between control subjects and ITP patients was significant

Table 31

Platelet volume distribution in 21 control subjects

Case no	Sex	Age	Platelet volume determination	Diagnosis	Platelet count ($x10^3/\mu l$)	Platelet volume		
						mean volume	mode log vol.	standard deviation log vol.
79	f	65	22-10-73	-	188	8.31	1.99	0.505
80	f	22	19-10-73	-	284	6.90	1.83	0.451
81	m	31	18-10-73	-	202	5.60	1.60	0.496
82	f	24	23-10-73	-	216	6.39	1.72	0.519
83	f	33	23-10-73	-	144	8.00	1.95	0.509
84	f	25	19-10-73	-	385	5.32	1.56	0.472
85	f	26	19-10-73	-	218	7.46	1.91	0.446
86	f	22	19-10-73	-	254	5.34	1.57	0.459
87	m	25	2-11-73	-	156	6.93	1.79	0.540
88	f	23	31-10-73	-	174	6.98	1.81	0.516
89	f	64	31-10-73	Carcinoma of ovary	316	5.70	1.63	0.470
90	m	49	31-10-73	Paraproteinemia	166	6.88	1.81	0.487
91	f	21	31-10-73	Anorexia nervosa	218	7.11	1.83	0.513
92	m	27	30-10-73	-	220	6.65	1.78	0.479
93	m	23	30-10-73	-	274	7.53	1.90	0.488
94	f	55	29-10-73	Carcinoma of breast	222	7.22	1.86	0.483
95	f	49	18-10-73	Hyperthyreoidism (treated)	297	5.83	1.63	0.516
96	f	29	17-10-73	-	156	7.37	1.87	0.505
97	m	42	29-10-73	Oesophagitis	272	7.22	1.86	0.483
98	m	38	26-10-73	-	188	7.58	1.90	0.501
99	m	27	30-10-73	-	220	7.19	1.86	0.475
Mean (n= 21)± SD					227 ±61	6.83 ±0.85	1.79±0.13	0.491±0.024

Table 32

Platelet volume distribution in 15 patients with (a history of) chronic ITP

Case no	Sex	Age	Platelet volume determination	Splenectomy	Prednison mg/day	Platelet count ($x10^3/\mu l$)	Platelet volume		
							mean volume	mode log vol.	standard deviation
1	m	42	24-10-73	+	-	329	6.26	1.71	0.498
2	m	20	26-10-73	+	-	137	8.48	2.03	0.442
3	f	35	24-10-73	+	-	307	7.69	1.94	0.447
4	f	28	22-10-73	-	-	170	7.25	1.84	0.531
5	f	56	29-10-73	+	-	87	10.05	2.14	0.579
6	m	60	23-10-73	+	-	66	8.81	2.06	0.481
7	f	67	29-10-73	+	-	240	9.17	2.10	0.482
8	m	27	24-10-73	+	20	44	9.00	2.09	0.463
9	f	60	29-10-73	+	-	42	7.80	1.88	0.590
10	f	25	23-10-73	+	-	174	10.35	2.21	0.504
11	f	26	26-10-73	+	-	165	8.39	2.02	0.463
14	m	30	1-11-73	+	-	87	9.84	2.15	0.522
15	f	33	18-10-73	+	-	414	6.55	1.77	0.468
			29-10-73	+	-	385	7.83	1.96	0.443
18	f	22	18-10-73	-	40	209	6.13	1.72	0.432
			25-10-73	-	15	310	5.55	1.60	0.477
22	f	28	29-10-73	-	-	111	9.18	2.06	0.560
Mean		(n=17)	± SD			193±120	8.14±1.44	1.96±0.18	0.493±0.048
Normal		(n=21)	± SD			227± 61	6.83±0.85	1.79±0.13	0.491±0.024

(p=0.003). The difference in the standard deviation of log volumes of platelets between ITP patients and control subjects was not significant. No correlation was found between platelet volume and blood platelet count or the age of the ITP patients. Only four patients with ITP were examined before splenectomy, and it was impossible to decide if the platelet volume of these patients differed from splenectomized patients.

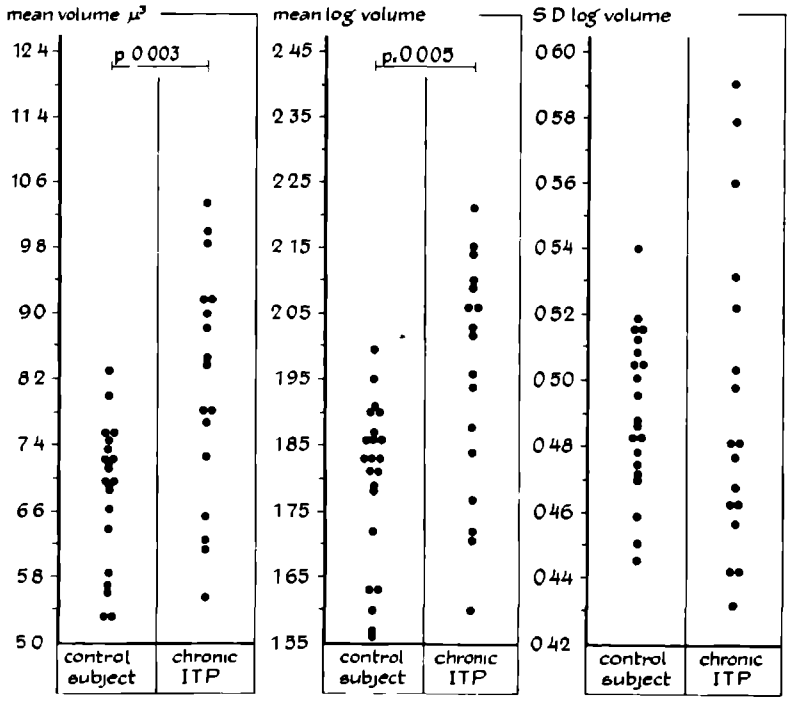

Fig. 26. Parameters of platelet size distribution in control subjects and patients with (a history of) chronic ITP

3.3.4. Discussion

The calculated mean platelet volume of 6.8 μ^3 in control subjects agrees with the findings of others (Bull et al. 1965; Karpatkin et al. 1969a,b; Garg et al. 1972; Paulus 1974). Only Paulus (1974) gives detailed information about platelet volume distribution by reporting the modes of the log volumes and standard deviations. In normal subjects he found a mode log volume of 1.38 to 1.86. In this study a range of 1.56 to 1.99 was found while the variation in platelet size in the individual subjects, as indicated by the standard deviation of log volumes of platelets, seemed to be smaller than that described by Paulus. In eight out of fifteen ITP patients the platelet size distribution was different from normal. The most important difference was the increased mean platelet volume. Only in 3 ITP patients was the variation in platelet size also increased. Our findings agree with other studies reporting an increased percentage of large platelets in ITP patients. In agreement with Garg et al. (1972) we also ob-

served patients with a history of ITP in apparent remission with an abnormal platelet volume distribution suggesting that there was still a stimulated thrombocytopoiesis caused by an increased platelet destruction compensated for by an adequate compensatory platelet production. In our study 5 patients were in a thrombocytopenic phase at the time of platelet volume distribution measurements. All of them showed an abnormal platelet volume distribution. The correlation between platelet volumes and the other observed parameters of thrombocytopoiesis and megakaryocytopoiesis will be discussed in chapter 4.

3.4. EFFECTIVE PLATELET PRODUCTION AFTER SPLENECTOMY

3.4.1. Introduction

While it is generally accepted that the spleen is an important site of platelet sequestration (Aster 1966, 1967; de Gabriele et al. 1967), the effect of the spleen on platelet production and the mechanism of postsplenectomy thrombocytosis is still unclear. After splenectomy, performed for various reasons, a thrombocytosis is observed. In theory postsplenectomy thrombocytosis may be due to one or more factors.

1. The removal of the splenic platelet pool (Ebbe 1968c; Krizsa et al. 1970).
2. Prolongation of platelet life span. However in the studies of Leeksma et al. (1965) in man, and of de Gabriele et al. (1967c) and Aster (1967) in animals, platelet life span was unchanged after splenectomy. Abrahamsen (1972) even observed a mild transitory shortening of platelet survival in the first week after removal of the spleen.
3. A shortened maturation time of megakaryocytes. Experimental work in animals gives no support for this explanation (Rolovic et al. 1970b).
4. A non-specific stimulus of platelet production due to the surgical procedure. This supposition must also be discarded because thrombocytosis after splenectomy is much more marked than after any other surgical procedure (Potts et al. 1941; Breslow et al. 1968).
5. A specific influence of the spleen on the rate of platelet production or on the rate of platelet destruction. The existence of such a humoral factor is suggested by experimental studies in animals in which reimplantation of a small portion of the spleen prevented postsplenectomy thrombocytosis (Tarnuzi et al. 1967). Shulman et al. (1965) suggested that this humoral factor does not inhibit the platelet production rate but increases platelet

destruction through the reticuloendothelial system. In this study we calculated platelet production before splenectomy retrospectively from the rise in postoperative blood platelet counts. It is well known that there is a lag period between a stimulus for thrombocytopoiesis and the rise in platelet counts. This lag period is explained by the fact that the stimulus for thrombocytopoiesis acts on the megakaryocyte precursor compartment or on the most immature megakaryocytes. The transit time of the stimulated cells through the recognizable megakaryocyte compartment causes this lag period. In human beings there are indications that this transit time is about 4 to 6 days (Cronkite et al. 1961; Muller 1967; Clarkson et al. 1971). If splenectomy were to influence the platelet production rate, a rise in platelet count should not occur during the first 4 to 6 days after removal of the spleen. For this reason the observed rise in blood platelet count during the first postoperative days must be explained on the basis of the presplenectomy platelet production level.
This supposition is confirmed by the findings of Harker and Finch (1969) in some thrombocytopenic patients with myelofibrosis and Hodgkin's disease. They observed that the preoperative estimation of platelet turnover agreed with the rate at which the peripheral platelet count increased during the first 4 days after splenectomy.
The postsplenectomy platelet production calculated from the peripheral platelet count will not reflect the platelet production before splenectomy if the platelet life span is altered. An increased platelet consumption postoperatively is reported by Abrahamsen (1972). This factor leads to an underestimation of platelet production but will probably be of the same importance in different kinds of splenectomized patients.

3.4.2. Materials and methods

In patients who were splenectomized for various reasons, postoperative platelet count levels were evaluated retrospectively. Only patients in whom, during the first 10 postoperative days, at least 4 platelets counts were performed, were analysed. Splenectomies were done in the years 1957-1973 in the surgical department (Head: Prof. Dr. W.J.H.Schmidt).

Examined subjects

Chronic ITP (24)

Twenty four patients with chronic ITP were examined. As earlier described under

the diagnostic criteria for chronic ITP the spleen weight in this group was normal.

M.Hodgkin (31)

A group of 31 patients with Hodgkin's disease contained patients in all stages of this disease. In none of them was the blood platelet count less than 100,000 /µl before splenectomy. In most cases the spleen was removed during explorative staging prqcedures in untreated patients. The spleen weight was often increased, mean 291 grams ± 123 (1 SD); with a maximum value of 530 grams.

Splenic rupture (11)

Eleven patients with traumatic injuries of the spleen were analysed. In some of them only a rupture of the spleen was diagnosed while in other patients there were also other traumatic injuries. In four cases the spleen was removed about one day after the accident. In the other cases there was a shorter interval between the accident and splenectomy.

Some clinical data regarding the three groups of splenectomized patients are given in table 33.

Table 33

Clinical data on patients in which platelet counts following splenectomy were analysed

	No. of patients			Age	Platelet count ($x10^3$/µl)
	male	female	total	mean ± SD	before splenectomy
chronic ITP	7	17	24	35 ± 17	22 ± 24
Hodgkin	19	12	31	32 ± 10	275 ±112
Splenic rupture	8	3	11	24 ± 12	?(normal:208±50)

Calculation of platelet production

Platelet production was calculated from the blood platelet counts of the first four postoperative days with a very simplified model. The following symbols are used in this model:

a = platelet count per µl before and at the time of splenectomy

b = platelet count per µl on the first postoperative day. C to n platelet counts per µl on the second to n postoperative days. The following assumptions and simplifications were made for the first four postoperative days:

1. After splenectomy all platelets delivered by the bone marrow are distributed through the total blood volume and there are no other platelet pools comparable with the spleen.
2. The increase in blood platelets postoperatively ((b-a), (c-a) etc.) is caused by postoperatively formed platelets.
3. From the platelets formed after splenectomy on the first postoperative day: 0.05(b-a) disappear, on the second postoperative day: 0.05(c-a) disappear etc.

   A disappearance rate of 0.05% was chosen for newly formed platelets in contrast with a disappearance rate of 0.10% for platelet population of a random age distribution.
4. From the presplenectomy formed platelets, with a random age distribution, 0.1 x a per µl disappear per day. If there was a fall in platelet count on the operative day, as often occurred in Hodgkin patients, 0.1 x b- was taken instead of -0.1 x a.
5. Platelet production per µl per day is the sum of the observed rise in blood platelet count and the calculated disappearance of the pre- and postsplenectomy formed platelets. This results on the first postoperative day in: (b-a) + 0.1 a + 0.05(b-a), on the second postoperative day in: (c-b)+ 0.1a + 0.05(c-a) etc.

In most of the ITP patients there was a very low platelet count before splenectomy. Therefore the influence of the presplenectomy formed platelets population (a) could be neglected.

3.4.3. Results

Mean values of platelet counts

Mean values of platelet counts on the various postoperative days in the three groups of patients are given in figure 27. The data for the 1st 4 days are based on 87 estimations of platelet levels in 24 patients with chronic ITP, 108 estimations in 31 patients with Hodgkin's disease and 33 estimations in 11 patients with traumatic splenic ruptures. In the later postoperative period less platelet counts were performed (table 34).

In patients with chronic ITP, platelet levels immediately after removal of the spleen show a continous marked rise, reaching a peak value of about $500 \times 10^3/\mu l$.

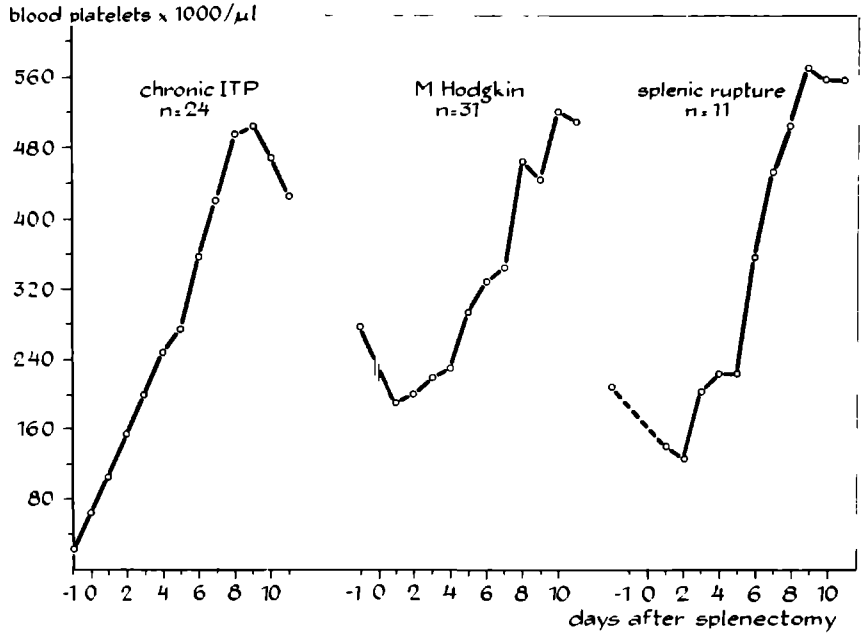

Fig. 27. Mean daily platelet levels after removal of the spleen in 24 patients with chronic ITP, 31 patients with Hodgkin's disease and 11 patients with traumatic splenic ruptures

The curves of platelet counts in splenectomized patients with Hodgkin's disease or traumatic spleen injuries show a biphasic course: a slow rate of platelet increase during 4 days is followed by a more striking rise. Another difference between the platelet levels after splenectomy in the three groups of patients is the postoperative dip seen in Hodgkin's patients and traumatic patients but not in the ITP patients. The mean daily platelet values in patients with splenic ruptures show an irregular course. In this group the number of examined patients and the number of platelet counts per patient were less than in the other groups. After the 5th day the course of the platelet counts in the three groups of patients was about the same.

Individual values of platelet counts

Generally the platelet count rise after splenectomy in chronic ITP patients was regular, while in patients with Hodgkin's disease or splenic ruptures a

Table 34

Pre- and postoperative platelet counts in 24 patients with chronic ITP, 31 patients with Hodgkin's disease and 11 patients with traumatic splenic ruptures

		Pre-	Post-operative day									
		0	1	2	3	4	5	6	7	8	9	10	11
chronic ITP	mean* 22	64	105	154	199	247	274	357	419	494	503	468	424
n:24	SD 24	33	80	111	128	144	198	247	316	285	304	241	238
	n 24	7	21	22	23	21	17	18	20	14	13	13	11
M.Hodgkin	mean 275		191	199	218	229	292	327	343	463	444	521	509
n:31	SD 112		81	80	104	90	126	144	115	201	171	211	206
	n 30		25	29	28	26	30	25	23	23	21	18	15
Splenic	mean(208*)		139	128	205	225	225	356	453	506	571	558	556
ruptures	SD (50**)		62	38	75	59	82	119	169	219	233	215	262
n:11	n		7	9	9	8	4	8	8	7	7	10	5

* mean platelet count ($x10^3/\mu l$)

** normal platelet count

period of slow increase during 4 to 7 days was followed by a striking increase. This node in the platelet count course never occurred before rhe 4th postoperative day. In 22 out of 24 patients with chronic ITP a significant rise in blood platelets was observed, reaching a peak 6to 13 days (mean 9 days) after splenectomy. In patients with Hodgkin's disease the postoperative peak was reached 7 to 17 days (mean 13 days)after splenectomy.

The calculated platelet production

The platelet production calculated in each patient from the blood platelet counts of the first four postoperative days resulted in a mean platelet production of 62 x $10^3/\mu l$/day (SD 45 x10^3) in patients with chronic ITP and 37 x $10^3/\mu l$/day (SD 33 x 10^3) in patients with Hodgkin's disease (fig. 28). This difference was significant (p=0.03; Wilcoxon's two sample test). In ITP patients and Hodgkin's patients there was no difference in calculated pla-

telet production between each of the first 4 postoperative days. However in Hodgkin's patients the platelet production on the fifth postoperative day was significantly higher than on the fourth day. In ITP patients platelet production on 4th and 5th day was equal. There was no correlation between the calculated platelet production and the blood platelet count before splenectomy or the age of the patients in either chronic ITP patients or in Hodgkin's patients. In patients with traumatic injuries of the spleen the number of platelet counts during the first 4 postsplenectomy days was insufficient for reliable calculation of platelet production.

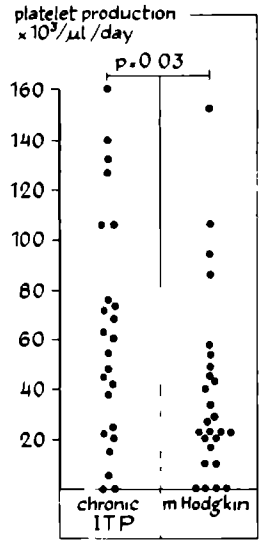

Fig.28. Platelet production calculated from the blood platelet counts during the first 4 days after splenectomy in 24 patients with chronic ITP and 27 patients with Hodgkin's disease

3.4.4. Discussion

The calculation of platelet production, with use of the simplified model for different disappearance rates of old and young platelets, is not free from criticism. The use of more sophisticated mathematical models in this study was not considered to be useful for the following reasons:

1. The influence of the surgical procedure on the consumption of platelets is in the first postoperative days much more important than the natural disappearance rate (Abrahamsen 1972).

2. The assumption of a normal platelet life span after splenectomy in ITP patients is, at least for some patients not valid.
3. The accuracy of routine platelet counts used in this retrospective study is rather low.

In patients with chronic ITP the calculated platelet production during the first four days after splenectomy was higher than in patients with Hodgkin's disease. In Hodgkin's disease platelet production will be normal or increased (Abrahamsen 1970, 1972). Therefore the difference in calculated platelet production between the ITP group with a mean value of 62 x 10^3/µl/day, and the Hodgkin group with a mean value of 37 x 10^3/µl/day suggests that the mean platelet production in ITP immediately after splenectomy is about twice the normal production. The individual calculations in ITP patients showed a maximum increase in platelet production of about 4 times the mean value in Hodgkin's disease. The difference in platelet production between patients with Hodgkin's disease and ITP will be underestimated because in our model a normal platelet life span was assumed in ITP patients after splenectomy. However, shortened platelet survival will occur in some ITP cases after splenectomy. One of our basic assumptions is that platelet production during the 1st 4 days after removal of the spleen will be the same as the platelet production before splenectomy. This was assumed because in man there is a lag period of about 5 days between the stimulus for a change in platelet production and the change in platelet delivery of the bone marrow into the blood (Cronkite et al. 1961; Müller 1967; Breslow et al. 1968; Clarkson et al. 1971). This was supported by the observed equal calculated platelet production in Hodgkin's disease before and during the fist days after splenectomy: immediately postoperative the calculated production was 36 x 10^3/µl/day; the presplenectomy blood platelet level of 275 x 10^3/µl also requires a daily platelet production of about 36 x10^3/µl/day assuming that the spleen pools 30% of all platelets and a platelet life span of 10 days. It therefore seems reasonable to assume that also in ITP patients the calculated platelet production immediately postoperative reflects the platelet production before splenectomy. As, moreover, we observed an increased platelet production in ITP immediately after splenectomy, this implies an increased platelet production in most chronic ITP patients before splenectomy.

Finally a general remark about postsplenectomy thrombocytosis:
A significant rise in calculated platelet production in Hodgkin patients from the fourth to fifth postoperative day was found, while in the same period in

patients with traumatic injuries a node in the course of the platelet counts was observed. It was calculated that this could not be explained by the removal of the splenic platelet pool and an unchanged platelet production, but suggests that splenectomy is a stimulus for an increased platelet production. This splenectomy-stimulus is more important than the stimulation of thrombocytopoiesis through other surgical procedures. So the old problem of the inhibitory function of the spleen on thrombocytopoiesis is still unsolved.

3.5. CONCLUSIONS

The observed normal thymidine incorporation by megakaryocytes in chronic ITP patients points to a normal DNA synthetic activity of the recognizable megakaryocytes. This observation, together with the normal frequency distribution of megakaryocytes in the various morphological maturation classes, suggest that maturation time of megakaryocytes in chronic ITP patients is normal. Pilot studies concerning the incorporation of uridine and methionine in vitro by megakaryocytes also gave indications for a normal activity of ITP megakaryocytes. So the morphological frequency distribution and the incorporation of thymidine, uridine and methionine argues against qualitative changes in megakaryocytopoiesis in chronic ITP patients. Platelet production calculated from the rise in blood platelet counts during the first days after splenectomy proved to be about twice the normal platelet production in chronic ITP patients. There are good arguments that this platelet production during the first days after splenectomy is identical to platelet production before splenectomy. The calculated increased platelet production in ITP suggests that the observed increase in platelet volumes in ITP is an indication of an increased platelet production and is not an indication of an abnormal thrombocytopoiesis. The observed change in platelet count and in calculated platelet production after splenectomy in non-ITP patients suggests that the maturation time of megakaryocytes is about 5 days.

Moreover the postsplenectomy thrombocytosis in these non-ITP patients can not be explained by the removal of the splenic platelet pool alone, but also suggests an inhibitory function of the spleen on thrombocytopoiesis.

CHAPTER 4

GENERAL DISCUSSION

As discussed in chapter 1 it is generally accepted that platelet survival is decreased in ITP. From the literature the influence of platelet production on the development of the thrombocytopenia in ITP is not clear. This seems to be due to the inaccuracy of calculating platelet production by methods using isotopically labelled platelets. In this study we tried to gain some insight into platelet production in ITP employing other methods: on the one hand by investigations on megakaryocytopoiesis and thrombocytopoiesis and on the other by calculating platelet production, using platelet counts immediately after splenectomy in ITP patients in comparison with other splenectomized subjects. The aspects of megakaryocytopoiesis and thrombocytopoiesis were studied in which, according to the literature concerning stimulated thrombocytopoiesis in human or animal experiments, differences might be expected. As described in chapters 1 and 2, the most relevant changes which may be expected in a stimulated thrombocytopoiesis are an increase in:

a. the total megakaryocyte volume
b. the mean individual megakaryocyte volume
c. the number of megakaryocytes
d. the ploidy level of megakaryocytes
e. the percentage of young (immature) megakaryocytes
f. the percentage of megakaryocytes with DNA synthetic activity
g. the platelet volume

In this study (3.1.3. and 3.2.3.) an increase in the percentage of young (immature) megakaryocytes and an increase in the percentage of megakaryocytes with DNA synthetic activity occurred only immediately following an acute stimulation of thrombocytopoiesis and not in chronic stimulation. Although this observation on first sight is in disagreement with the literature, the normal percentage of immature megakaryocytes was easily explained by the duration of the stimulation of thrombocytopoiesis in chronic ITP patients. The normal DNA synthetic activity of megakaryocytes in patients with chronic ITP can also be explained by the duration of the stimulation and by the fact that the observed ploidy level

of megakaryocytes in chronic ITP was only moderately increased (3.1.4.).The group of patients with chronic ITP studied, showed an increased (p=0.03) platelet production (3.4.3.) and the other changes expected in a stimulated thrombocytopoiesis (excluding the 2 above mentioned parameters):

a. the total megakaryocyte volume in marrow biopsies was on the average 1.9 times greater (p=0.0001) than normal (2.2.2.).
b. the individual megakaryocyte volume was greater than normal as demonstrated by the diameter of sections of megakaryocytes which was on the average 1.2 times increased (p= 0.001) and by the mean area of sections of megakaryocytes which was on the average 1.1. times increased (p= 0.04).
c. the number of megakaryocytes was increased (2.2.3.).
d. the mean ploidy level of megakaryocytes was 1.1 times greater than normal (p=0.06) (2.2.2.).
g. the mean platelet volume was 1.2 times greater (p= 0.003) than normal (3.3.3).

So an increased platelet production and changes in megakaryocytopoiesis and thrombocytopoiesis, as expected in a stimulated thrombocytopoiesis, were found in the groups of ITP patients. This does not mean that the increase in platelet production is as much as might be expected from the increased potential platelet production capacity. The observation in the groups of patients with ITP shows that there is a quantitative agreement between the increase in produced total platelet volume and the increase in total megakaryocyte volume, since the total megakaryocyte volume increase was 1.9 times, the increase in the number of produced platelets was 1.7 times and the increase in the mean platelet volume was 1.2 times. Despite this quantitative conformity of static parameters, thrombocytopoiesis might be ineffective if the turnover rate of megakaryocytes is greater than normal. However in this study evidence was found that indicated that the maturation time of megakaryocytes is not shortened when thrombocytopoiesis is stimulated:

a. In non-ITP patients an increase in platelet production was found after splenectomy (3.4.2.). Despite this stimulation of thrombocytopoiesis a normal megakaryocyte maturation time of about 5 days was found.
b. The normal percentage of megakaryocytes of type I combined with the normal thymidine labelling of megakaryocytes in patients with chronic ITP also suggest a normal maturation time (3.2.4.).

Another argument for a normal megakaryocyte maturation time despite a stimulation of thrombocytopoiesis can be deduced from the observations of Harker et al. (1969) and Kutti et al. (1973). They found a direct, linear relationship between the total megakaryocyte volume and platelet production. If the maturation

time of the megakaryocytes is increased in stimulated thrombocytopoiesis one would expect that the increase in the total megakaryocyte volume would be less than the increase in platelet production. As well was these quantitative agreements between platelet production capacity (total megakaryocyte volume) and platelet production there is still another argument against an ineffective thrombopoiesis in ITP in patients with ineffective thrombocytopoiesis the mean (individual) megakaryocyte volume is normal or decreased (Harker et al. 1969). But in patients with chronic ITP an increased megakaryocyte size has been found by Harker et al. (1969) and again in this study.

The lack of indications for an ineffective thrombocytopoiesis does not exclude abnormalities in megakaryocyto- and thrombocytopoiesis. That is to say, it cannot be excluded that megakaryocytes may be destroyed before they can release platelets particularly since antibodies have been demonstrated on megakaryocytes from some ITP patients (Pisciotta et al. 1953, Mac Kenna (1962). If these antibodies led to a destruction of megakaryocytes one would expect that specifically the mature megakaryocytes, which have many antigens in common with platelets, would be destroyed. However in this study a normal percentage of mature, type III, megakaryocytes have been found and Harker et al. (1969) found a normal cytoplasm-nucleus ratio in megakaryocytes from ITP patients.

To summarise; the results of our investigations in groups of patients with (a history of) ITP support the idea of an increased platelet production in ITP which is quantitatively in conformity with the observed increase in platelet production. The results from the groups of ITP patients gave no indications of an ineffective thrombocytopoiesis or disturbance of megakaryocyto- or thrombocytopoiesis. The results of the described investigations make a partial destruction of megakaryocytes appear unlikely, although this cannot be excluded with certainty.

After this discussion of the results from the groups of patients with ITP, the results in the individual patients will be discussed. The results of the various parameters of megakaryocytopoiesis and thrombocytopoiesis in the individual patients are given in the appendix .Patients with ITP showed a clear overlap of normal and increased values (2.2.2., 3.1.3., 3.2.3., 3.3.3. and 3.4.3.) as is summarized in table 35. Of these parameters megakaryocyte volume, the mean section diameter and the mean platelet volume were the three parameters which were most frequently abnormal in ITP. An attempt was made to classify

ITP patients by use of these three parameters. In table 36 the values of these parameters in patients with ITP are arranged according the severity of the thrombocytopenia and the determined degree of stimulation of thrombocytopoiesis at the date of the bone marrow examination. The degree of stimulation of thrombocytopoiesis was indicated and evaluated as:

+++ : platelet count less than 40,000/µl,

++ : platelet count 40,000 - 80,000 /µl,

+ : platelet count 80,000 -110,000 /µl, a normal platelet count unless there was a shortened platelet life span or a normal platelet count and an offspring with neonatal thrombocytopenia,

- : normal platelet count and normal platelet life span (determined by isotopic labelling of platelets)

? : normal platelet count but no determination of platelet life span available.

Table 35

Number of increased and normal values for parameters of megakaryocytopoiesis and thrombocytopoiesis in patients with ITP

	Total number of investigations	Number of values	
		above the normal range	below the normal range
total megakaryocyte volume	24	18	0
mean megakaryocyte volume (section diameter)	24	15	0
mean megakaryocyte ploidy	14	3	0
mean platelet volume	17	7	0
calculated platelet production	24	6	0

21 marrow biopsies were taken during stimulation of thrombocytopoiesis. In these the total megakaryocyte and the mean section diameter were increased 16 and 13 times respectively (table 36). In one splenectomized patient with a history of ITP a stimulation of thrombocytopoiesis seems unlikely because the platelet count and platelet life span were normal. In this patient the total megakaryocyte volume, the mean section diameter and the mean platelet volume were normal, indicating a complete remission after splenectomy. In two

Comparison of the degree of stimulation of thrombocytopoiesis and the results of determinations of total megakaryocyte volume, mean section diameter of megakaryocytes and mean platelet volume in patients with (a history of) ITP

Case no.	Platelet count ($x10^3/\mu l$) at the time of marrow examinations	Stimulation	Megakaryocyte total volume	Megakaryocyte mean section diameter	Platelet count at the time of platelet volume examination	Stimulation	Mean platelet volume
8	2	+++	=	=	44	++	↑
17	7	+++	↑	↑			
13	8	+++	↑	↑			
16	10	+++	↑	↑			
14	10	+++	↑	↑	87	+	↑
15	10	+++	↑	=	385	?	=
					414	?	=
1a	16	+++	↑	=	329	-	=
12	23	+++	↑	↑			
18	35	+++	↑	↑	209	?	=
					310	?	=
9	48	++	↑	=	42	++	=
6a	55	++	↑	=	66	++	↑
6b	58	++		=			
7a	56	++	=	↑	240	?	↑
6c	66	++	↑	↑	66	++	↑
5a	92	+	↑	=	87	+	↑
4	92	+	↑	↑	170	?	=
2	110	+	=	↑	137	?	=
11	166	+	↑	↑	165	?	=
3	170	+	↑	=	307	?	=
10a	200	+	=	↑	174	+	↑
10b	205	+	=	↑			
5b	156	?	↑	↑	87	+	↑
7b	322	?	↑	↑	240	?	↑
1b	268	-	=	=	329	-	=
22					111	?	↑

splenectomized patients in which stimulation of thrombocytopoiesis had not been studied the increased total megakaryocyte volume and mean section diameter suggested that after splenectomy an increased platelet destruction was compensated by an increased platelet production. A determination of platelet volume was performed 6 times in patients in whom thrombocytopoiesis was thought to be stimulated (table 37). In only one of them was no increase in the mean platelet volume observed (no.9). However in this splenectomized patient the variation in platelet size was much greater than normally. Moreover this was a peculiar patient because

Table 37

Comparison of the degree of stimulation of thrombocytopoiesis and the mean platelet volume in patients with a history of ITP

At the time of platelet volume estimation

Case no.	Platelet count ($\times 10^3/\mu l$)	Stimulation	Mean platelet volume
9	42	++	=
8	44	++	↑
6 ab	66	++	↑
5 b	87	+	↑
14	87	+	↑
22	111	?	↑
2	137	?	=
11	165	?	=
4	170	?	=
10 ab	174	+	↑
7 a	240	?	↑
3	307	?	=
1 a	329	-	=
15	385	?	=
15	414	?	=

the platelet life span was normal in spite of a thrombocytopenia. In patients with a history of ITP with dubious stimulation of thrombocytopoiesis the platelet volume varied between 2 and 8 times normal. Of the 2 patients with an increased platelet volume one patient also showed an increased total megakaryocyte volume suggesting a compensated thrombocytolysis while in the other patient, whose total megakaryocyte volume was not determined, the low borderline value for blood platelet counts (111,000/µl) also suggested a stimulated thrombocytopoiesis. In six patients with a stimulated thrombocytopoiesis, values of all three parameters (total megakaryocyte volume, the mean section diameter and platelet volume), were together available nine times. On two occasions all three parameters were increased, five times two were increased, and twice only one of the parameters was increased. So in cases with a stimulated thrombocytopoiesis the total megakaryocyte volume, the mean section diameter and the mean platelet volume are generally increased but sometimes normal. Therefore several explanations are possible:

1. changes in all three parameters occur dependent on the degree of stimulation of thrombocytopoiesis. It is conceivable, for instance, that moderate stimulation only causes an increase in the number of megakaryocytes. This will result in an increase in the total volume of megakaryocytes but not in an increase in

the mean section diameter of megakaryocytes as has been observed in some cases in this study. So it is also conceivable that the only result of a moderately stimulated thrombocytopoiesis is an alteration in platelet volume or megakaryocyte volume. However in this study no correlation was found between the degree of the thrombocytopenia and the frequency of increase in total megakaryocyte volume, section diameter or platelet volume. There was also no correlation between the degree of the thrombocytopenia and the degree of increase of the various mentioned parameters.

2. It is possible that after splenectomy the normal value for total megakaryocyte volume is 30% less than before splenectomy (2.2.3.). If this is true then the value for the total megakaryocyte volume in patients with stimulated thrombocytopoiesis is increased in all but one patients and there is no longer a discrepancy between the increased mean section diameter and the normal total megakaryocyte volume in the three splenectomized ITP patients.

3. With the techniques employed, moderate alterations in the parameters used to recognize stimulated thrombocytopoiesis are undetectable.

4. The parameters of stimulated thrombocytopoiesis are influenced by other factors. E.g. it is conceivable that antiplatelet antibodies led to alterations in the individual megakaryocyte volume and/or to destruction of megakaryocytes. Arguments against this hypothesis have been mentioned above.

5. Various pathogenic factors exist. In this case ITP should be considered as a clinical syndrome with various pathogenetic factors which lead to various alterations in megakaryocytopoiesis and thrombocytopoiesis. However, there are too many combinations of normal and increased values of total megakaryocyte volume, individual megakaryocyte volume and platelet volume to classify ITP patients into a series of logical subgroups.

A classification of ITP patients into various types is not facilitated by consideration of the mentioned parameters together with other characteristics such as age, sex, course of the thrombocytopenia (neither spontaneous nor following treatment with corticosteroids or splenectomy). A classification of patients with ITP into patients with ineffective and patients with effective thrombocytopoiesis is also not possible because only in some patients, for whom the total megakaryocyte volume was known, could the platelet production also be calculated (immediately after splenectomy). Moreover the values for the calculated platelet production, although useful for comparing groups of patients, are too inaccurate for individual patients because complications such as postoperative infections and haemorrhages influence the calculated production. So the analysis of

the various characteristics of patients with ITP does not lead to a logical classification into sub-groups.

Our observations suggest that the effect of splenectomy is less favourable in patients with ITP in whom the total megakaryocyte volume is not clearly increased. The number of observations is still small. Therefore at this moment we can not advise against splenectomy in patients with ITP when the total megakaryocyte volume is not increased. However, the finding of a normal total megakaryocyte volume in ITP patients should lead to more conservative management: treatment with corticosteroids and immunosuppressive agents should be carried out more extensively and relative contra-indications for splenectomy should be accorded more weight.

If the total megakaryocyte volume is decreased in patients with the diagnosis ITP, then this diagnosis should be reconsidered because in this study it has never been observed in undisputed ITP patients.

SUMMARY

From the literature the role of platelet production in the pathogenesis of a thrombocytopenia in patients with chronic idiopathic thrombocytopenic purpura (ITP) is not clear. This study concerns the level of platelet production in ITP which has been examined by investigating some aspects of megakaryocytopoiesis and thrombocytopoiesis.

In chapter 1 the literature on megakaryocytopoiesis, thrombocytopoiesis and platelet kinetics under normal and pathological conditions is briefly reviewed. Special attention has been paid to phenomena suggesting a stimulating thrombocytopoiesis because such parameters have been thought to be valuable indications for an increased platelet production in ITP.

Chapter 2 deals with the method for examination of the productivity of megakaryocytes by a study of their number and their individual volumes; the total megakaryocyte volume in marrow biopsies as an indication of platelet production capacity. From the literature and our own observations, it is clear that determinations of the number of megakaryocytes in bone marrow aspirates, especially in marrow smears, are unreliable. Attention has been directed towards the importance of the individual volume of megakaryocytes for their platelet production capacity. Using the described histometric method, the product of the number of megakaryocytes and their volumes in marrow biopsies has been determined. The results of these histometric determinations in 18 patients with chronic ITP were significantly increased in comparison with 14 control subjects, while in patients with a thrombocytopenia caused by bone marrow hypoplasia lower figures were obtained. In some patients with a history of ITP but normal platelet counts, an increase in total megakaryocyte volume was found suggesting that platelet destruction was still increased but was compensated by an increased platelet production. Diagnosis of such compensated thrombocytolysis proved to be of practical importance in three pregnant women with a history of ITP but with normal platelet counts. This information makes it possible to predict that the offspring of such patients may show a thrombocytopenia

which could be important for the management of the delivery. The increase in total megakaryocyte volume in ITP is caused by an increase in the number, as well as in the individual volumes, of megakaryocytes. Attention is directed towards the importance of cellularity of the bone marrow biopsy in judging the significance of the total megakaryocyte volume of a marrow biopsy for the total megakaryocyte volume of the body.

Moreover in chapter 2 the observed increase in ploidy levels determined by microfluorometric measurements of Feulgen stained nuclei in patients with chronic ITP is discussed. This increase is in accordance with the increase in the individual megakaryocyte volume in ITP and will result in an increase in the platelet production capacity of the individual megakaryocytes.

While chapter 2 deals with some static aspects of megakaryocyto- and thrombocyto-poiesis, chapter 3 deals with some dynamic aspects . namely metabolic and functional aspects of megakaryocyto- and thrombocytopoiesis. After in vitro incubation of megakaryocytes with radioactive thymidine, the thymidine uptake, as studied by autoradiography, proved to be normal in 12 patients with chronic ITP. On the other hand, in 5 out of 6 patients with an acute stimulation of thrombocytopoiesis, the labelling index of megakaryocytes was increased. Pilot autoradiographic studies concerning ^{3}H-uridine and ^{3}H-methionine uptake suggest that there are no important alterations in RNA- and protein synthesis-activity in ITP patients. Autoradiographs were prepared using a special technique in order to conserve the morphology of May Grunwald Giemsa stained cells. By this means it was possible to classify megakaryocytes in autoradiographs into various maturation classes. This classification was normal in those patients studied, who had chronic ITP while in 2 patients with an acute post-operative thrombocytopenia an increase in the number of immature, type I, megakaryocytes was observed. The reasons for considering the combination of normal distribution of megakaryocytes in their various maturation classes and a normal thymidine labelling index of megakaryocytes in chronic ITP patients, as an indication of a normal megakaryocyte maturation time are discussed.

The platelet volume proved to be increased in patients with chronic ITP. The significance of this finding for stimulated or abnormal thrombocytopoiesis is discussed.

Finally, in chapter 3 it is demonstrated that the mean platelet production in 24 patients with ITP is about twice that of 42 patients with Hodgkin's disease or traumatic splenic ruptures. This comparison has been made by calculating platelet production from blood platelet counts immediately after splenectomy. From these observations it can, moreover, be concluded that postsplenectomy thrombocytosis in non- ITP patients is not only due to removal of the spleen, as is often asserted, but is also partly caused by an increased platelet production which becomes evident about five days after splenectomy. Those five days are seen as the time needed for maturation of the megakaryocytes and so this supports our conclusion that the maturation time of human megakaryocytes is not, or is only slightly, increased if thrombocytopoiesis is stimulated.

In chapter 4 the results described in the foregoing chapters are brought together and analysed. The mean increase in the total megakaryocyte volume in marrow biopsies in the group of ITP patients studied, is quantitatively in accordance with the mean increase in calculated platelet production observed in a retrospective study of a group of patients with ITP, which also includes another group of ITP patients. Since in this study no indications have been found for a shortened megakaryocyte maturation time when thrombocytopoiesis is stimulated, the quantitative agreement between the increase in total megakaryocyte volume and the increase in platelet production suggests that thrombocytopoiesis is effective in ITP.

Using the parameters of megakaryocytopoiesis and thrombocytopoiesis which are most frequently abnormal (increased) in patients with ITP together with the patients clinical characteristics, an attempt is made to classify patients with ITP into different types. In most patients the findings suggest an increased platelet production. In some patients no adequate reaction of the bone marrow to the thrombocytopenic stimulus seems to occur. This is accompanied by unsuccesful splenectomy. However such patients were rare and there were many transitional stages between them and ITP patients with signs of an increased thrombopoietic activity in whom splenectomy was succesful. Therefore a logical classification of ITP patients based on the parameters employed in this study was not possible.

Appendix

Survey of investigations in all examined patients. The results of the determinations the mean normal values.

Case no.	Age	Sex	Date of examination	Splenectomy	Prednison (mg/day)	Platelet count ($x10^3/\mu l$)	Total volume X		N **		Mean section diameter X		N		No. of sections X		N		Mean ploidy X		N		% type I X		N		Thymidine labelling X		N	
1	41	m	06-07-72	-	-	16	2.0	↑	1.0	=	1.6	↑	1.2	=	0,9	=	0,7	↑
			08-01-73	+	-	268	1.1	=	1.0	=	0.8	=						
			04-06-73	+	-	213							1.1	=				
			24-10-73	+	-	329												
2	19	m	29-11-72	+	-	110	1.4	=	1.2	↑	1.2	=						
			26-10-73	+	-	137												
3	34	f	11-10-72	+	-	170	1.7	↑	1.1	=	1.3	=						
			24-10-73	+	-	307												
4	27	f	12-01-73	-	-	92	1.8	↑	1.3	↑	1.5	=						
			22-10-73	-	-	170												
5	55	f	28-07-72	-	-	38							1.2	=	0.9	=	1.1	=
			18-10-72	-	30	92	1.9	↑	1.1	=	1.8	↑						
			25-01-73	+	-	156	1.8	↑	1.3	↑	1.3	=						
			29-10-73	+	-	87												
6	59	m	04-09-72	-	60	29							1.0	=	1.6	=	0.9	=
			07-09-72	-	60	58	1.7	↑	1.1	=	1.7	↑						
			01-02-73	+	-	55	2.3	↑	1.0	=	1.6	↑						
			20-09-73	+	-	66	1.8	↑	1.4	↑	1.8	↑						
			23-10-73	+	-	66												
7	65	f	10-04-72	-	-	16									0.7	=	1.2	=
			04-05-72	-	25	56	1.1	=	1.2	↑	1.1	=			0.8	=	1.2	=
			06-72	-	-	15												
			05-07-72	+	10	184									0.8	=	0.9	=
			18-01-73	+	-	322	1.6	↑	1.3	↑	1.1	=						
			29-10-73	+	-	240												
8	26	m	14-01-72	+	40	94									0.8	=	1.0	=
			19-06-72	+	20	2	1.1	=	1.1	=	1.1	=	1.3	↑	1.0	=	0.8	=
			24-10-73	+	20	44												
9	59	f	01-73	+	-	43												
			13-06-72	+	-	48	1.9	↑	1.1	=	2.5	↑						
			29-10-73	+	-	42												
10	24	f	10-04-72	+	-	220									1.0	=	0.9	=
			09-72	+	-	193												
			18-12-72	+	-	205	1.4	=	1.3	↑	1.2	=						
			12-06-73	+	-	200	1.3	=	1.2	↑	1.1	=						
			23-10-73	+	-	174												

of the various megakaryocytes and platelet parameters are expressed as functions of

platelet							
^{3}H-methionine	Mean platelet volume XN	Platelet production XN	Platelet half-life time (^{51}Cr of $DF^{32}P$) XN	Date of splenectomy	Results of splenectomy*	Case no.	Remarks
+	0.9 =	2.0 =	1.0 =	26-10-72	Good	1	ITP
	1.2 =		0.6 ↓	1968	Good	2	ITP
	1.1 =			1968	Good	3	ITP;offspring with neonatal thrombocytopenia 09-03-73
	1.1 =		0.2 ↓			4	ITP
+	1.5 ↑	1.9=	0.7 ↓	26-10-72	Moderate	5	ITP
+	1.3 ↑	0.1=	0.3 ↓	11-10-72	Moderate	6	ITP Postsplenectomy respiratory infection
	1.3 ↑		<0.1 ↓	27-06-72	Good	7	ITP
+	1.3 ↑	1.0=	0.1 ↓	14-12-71	Poor	3	ITP
	1.1 =		1.0 =	1947	Poor	9	ITP
	1.5 ↑	0.1=	0.6 ↓	13-09-71	Poor	10	ITP; remission with azathioprine offspring with neonatal thrombocytopenia, 05-11-73

Appendix (continued)

Case no.	Age	Sex	Date of examination	Splenectomy	Prednison	Platelet count ($\times 10^3/\mu l$)	Megakaryocytes: Total volume X	N	**	Mean section diameter X	N		No.of sections X	N		Mean ploidy X	N		% type I X	N		X	N
11	26	f	20-02-73	+	-	166	2.1		↑	1.2		↑	2.1		↑	1.3		↑					
			26-10-73	+	-	165																	
12	45	f	17-04-72	+	5	16										1.0		=	1.4		=	1.1	=
			28-08-72	+	10	23	3.2		↑	1.2		↑	2.6		↑								
13	65	f	13-03-72	+	5	14										1.1		=	0.8		=	0.9	=
			22-06-72	+	5	8	1.6		↑	1.2		↑	1.2										
14	30	m	11-0-73	-	-	10	4.1		↑	1.3		↑	3.4		↑	1.1		=					
			03-03-73	+	80	188										1.1		=	0.5		=	0.5	↓
			01-11-73	+	-	87																	
15	33	f	04-07-73	+	30	10	2.1		↑	1.1		=	1.5		=								
			18-10-73	+	-	414																	
			29-10-73	+	-	385																	
1(	49		21-05-73	+	1(	0	1.8		↑	1.2		↑	1.7		↑								
1				·	-	7	2.2		↑	1.2		↑	2.2		↑								
18	22	f	04-10-73	-	-	35	1.7		↑	1.2		↑	2.2		↑	0.8		=					
			18-10-73	-	40	209																	
			25-10-73	-	15	310																	
19	13	m	28-09-72	-	-	20										1.1		=	0.7		=	1.0	=
2			05-52	-	15	80										1.3		↑	1.0		=	1.3	=
2			)3-73	+	-	262													0.7		=	1.0	=
2			)-73	-	-	111																	
2	48	f	17-12-72	-	-	30	0.2		↓				0.3		↓								
2	53	f	28-08-72	-	-	50	0.7		=				0.5		=								
25	18	f	12-09-72	-	-	27	0.4		↓				0.4		↓								
			19-09-73	-	-	34	0.4		↓				0.4		↓								
26	19	f	18-09-72	-	-	19	0.3		↓				0.3		↓								
27	23	f	04-72	+	40	10																	
			19-06-72	+	5	15	1.7		↑				1.5		=								
28	62	f	23-10-72	-	-	84	1.1		=				1.2		=								
29	32	f	11-72	-	-	122																	
			18-01-73	-	-	120	1.3		=				1.1		=								
			08-03-73	-	-	113	1.4		=				1.3		=								

^{3}H-methionine	platelet Mean platelet volume	platelet production	platelet half-life time (^{51}Cr-or DF^{32}P	Date of splenectomy	Results of splenectomy*		Remarks
	X N	X N	X N				
	1.2 =			1954 24-02-72	Good 1970 recidive	11	ITP; offspring with neonatal thrombocytopenia,19-11-71; removal of accessory spleen, 24-02-72
				1957	Poor	12	ITP
				1940 1966	Poor Poor	13	ITP 1966, removal of accessory spleen
		1.7 =		28-02-73	Moderate	14	ITP
	1.4 ↑						
				1955	Good	15	ITP; intermittent thrombocytopenia
	1.0 =						
	1.1 =						
				28-11-72	Poor	17	ITP
						18	ITP
	0.9 =						
	0.8 =						
+	+					19	ITP
+	+			1972	Good	20	ITP
				14-10-71	Good	21	ITP
	1.3 ↑					22	ITP
						23	Hypoplastic thrombocytopenia
						25	Hypoplastic thrombocytopenia
						26	Hypoplastic thrombocytopenia
			< 0.1 ↓	06-04-72	Poor	27	Lupus erythematosus
						28	Drug induced? Thrombocytopenia (gold)
			0.8 =			29	Thrombocytopathy

Appendix (continued)

Case no.	Age	Sex	Date of examination	Splenectomy	Prednison	Platelet count ($x10^3/\mu l$)	Megakaryocytes: Total volume X N**	Mean section diameter X N	No. of sections X N	Mean ploidy X N	% type I X N	Thymidine labelling X N
30	26	f	15-09-73	-	-	50	1.5 =		1.6 ↑			
31	13	f	27-09-73	-	-	110	1.1 =		1.0 =			
32	46	f	10-05-73	-	-	1031	6.8 ↑		3.8 ↑			
33	64	m	24-05-72	-	-	850	3.3 ↑		2.8 ↑	1.5 ↑		
34	58	f	29-06-72	-	-	686	1.7 ↑		1.2 =			
			07-72	-	-	750						
35	34	f	29-03-72	-	-	980	6.9 ↑		4.2 ↑			
36	53	m	23-11-72	-	-	673				0.6 ↓		
37	61	f	12-07-73	-	-	295				0.8 =		
38	43	m	02-03-73	-	-	83				0.8 =	1.7 ↑	1.9 ↑
39	49	m	29-03-73	-	-	80				1.1 =	1.0 =	1.6 ↑
40	30	m	29-03-73	-	-	64				1.0 =	1.3 =	1.2 =
′		?	01-12-72	-	-	70					2.1 ↑	1.8 ↑
′											1.5 =	1.8 ↑
4											1.4 =	1.5 ↑
44	23	m	23-02-72	-	-	124					0.9 =	0.5 ↓
			03-72			158						
′			04-72	+	-	80					0.8 =	0.7 ↓
46	60	m	19-04-72	-	-	152					1.2 =	1.6 ↑
47	17	m	12-04-72	-	-	100					0.8 =	1.0 =
			04-05-72	-	-	309					0.8 =	0.9 =

*Results of splenectomy; good if platelet count > 100,000/µl; moderate if platelet

**X N= function of the mean normal value. Values in the normal range (mean ± SD) are

^{3}H-methionine	Mean platelet volume X N	Platelet production X N	Platelet half-life time (^{51}Cr-DF ^{32}P) X N	Date of splenectomy	Results of splenectomy *	Case no.	Remarks
						30	fam. thrombocytopenia
						31	fam. thrombocytopenia
						32	primary thrombocytosis
						33	urticaria pigmentosa, thrombocytosis
			1.0 =			34	rheumatoid arthritis, thrombocytosis
			0.6 ↓			35	myelofibrosis
						36	chronic myeloid leukaemia
						37	chronic myeloid leukaemia
						38	atrial septal defect
						40	insufficiency
						41	atrial septal defect
						42	mitral insufficiency
						43	prosthetic hip surgery
						44	auto-immune haemolytic anaemia, splenomegaly
			0.6 ↓				
				)5-71	Moderate	45	lupus erythematosus
						46	infectious thrombocytopenia
						47	thrombotic thrombocytopenic purpura?

post operative thrombocytopenia

)unt= 50,000-100,000/µl; poor if platelet count: < 50,000/µl

ce indicated by =. Values falling outside of the normal range are indicated by ↑ or ↓.

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CURRICULUM VITAE

Schrijver van dit proefschrift werd geboren op 3 februari 1936 te Alkmaar. Hij bezocht vanaf 1948 het Petrus Canisius Lyceum te Alkmaar tot in 1954 het HBS-B examen werd behaald.

De studie in de geneeskunde vond plaats aan de Katholieke Universiteit te Nijmegen. In 1960 werd het doctoraalexamen afgelegd. De opleiding tot arts werd voortgezet aan de Stichting Klinisch Hoger Onderwijs te Rotterdam, waar in 1962 het artsexamen van de Rijksuniversiteit te Leiden werd behaald.

De militaire dienst werd vervuld als docent aan de School voor Reserve Officieren van de Militair Geneeskundige Dienst te Amersfoort.

Van september 1964 tot juli 1974 was hij verbonden aan de Universiteitskliniek voor Inwendige Ziekten te Nijmegen (directeur Prof. Dr C.L.H.Majoor). Na het beëindigen van de opleiding tot internist met de aantekening rontgenologie op 1 september 1969 was hij voornamelijk verbonden aan de afdeling voor hematologie (hoofd Prof. Dr C.A.M.Haanen).

Sinds 1 juli 1974 is hij werkzaam als internist aan het Catharinaziekenhuis te Eindhoven.

STELLINGEN

1

Schattingen van het aantal megakaryocyten in uitstrijkpreparaten van een beenmergpunctaat zijn onbetrouwbaar.

Dit proefschrift

2

Bij het beleid van een partus bij vrouwen met een ziektegeschiedenis van chronische idiopathische trombocytopenische purpura dient men bedacht te zijn op een verhoogde bloedingsneiging van het kind, ook indien het trombocytenaantal bij de moeder normaal is.

Dit proefschrift

3

Pokkenvaccinatie van zuigelingen is in Nederland zinloos.

Dekking, F.: Ned. T. Geneesk. 117, 1903, 1973.

4

Bij het verrichten van kruisproeven dient naast de kruisproeven in fysiologisch zout en naast de indirecte antiglobulinetest ook een kruisproef te worden verricht waarbij de erythrocyten met een eiwitsplitsend enzym zijn voorbehandeld.

Kunst, V.A.J.M., Bloo, J.H., Kruytzer, G.P.J., Mc Shine, R.L. en Reekers, P.P.M.: Ned. T. Geneesk., ter perse.

5

De diagnose paradoxe splijting van de tweede toon kan men auscultatoir alleen stellen indien bij inspiratie en expiratie 2 componenten hoorbaar zijn.

Lucardie, S.M.: Ned. T. Geneesk. 11, 2005, 1967.

6

De behandeling van idiopathische proctitis met acetarsol suppositoria geeft betere resultaten dan die met prednisolon.

Connel, A.M. Lennard-Jones, J.E., Misiewicz, J.J., Baron, J.H., Avery-Jones, F.: Lancet, 1, 238, 1965.

7

Het lichamelijke prestatievermogen, getest met behulp van de fietsergometer, moet worden gerelateerd aan de hoeveelheid vetvrij weefsel.

Leusink, J.A.: Dissertatie, Utrecht, 1972.
van Reekum, J.R.: Dissertatie, Utrecht, 1974.

8

Wanneer men overweegt patiënten met een hypo-immunoglobulinemie te behandelen met immuunglobulinen dient eerst een onderzoek naar de aanwezigheid van circulerende anti-IgA antistoffen plaats vinden.

Vyas, G.N., Perkins, H.A. en Fudenberg, H.H.: Lancet 2, 312, 1968.
Nadorp, J.M.S.M.: Dissertatie, Nijmegen, 1974.

9

Bij verdenking op een arterioveneuze misvorming van de bloedvaten van het ruggemerg is de spinale angiografie de meest aangewezen methode om de afwijking aan te tonen en zo volledig mogelijk te lokaliseren.

Di Chiro, G en Wener, L.: J. Neurosurg., 39, 127, 1973.

10

Bij langdurige behandeling met orale anticoagulantia van het coumarinetype verdient het gebruik van phenprocoumon (Marcoumar) de voorkeur boven acenocoumarol (Sintrom).

Breed, W.P.M., van Hooff, J.P. en Haanen, C.: Acta Med. Scand. 186, 283, 1969.

11

Vitamine D is geen vitamine.

Stamp, T.C.B.: Arch. Dis. Child., 48, 2, 1973.